University of Agronomic Sciences and Veterinary Medicine of Bucharest
Faculty of Animal Productions Engineering and Management

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TESTING GENETIC ASSOCIATIONS OF THE SNP C.1053C>T POLYMORPHISM FROM DGAT1 GENE WITH MILK QUALITATIVE PARAMETERS IN RIVER BUFFALO (*Bubalus bubalis*)

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Abstract

Improving milk yield and milk composition are objectives of interest in the selective breeding of animals. Milk fatty acids and proteins are important in the manufacturing of many buffalo dairy products, the best-known being mozzarella cheese. DGAT1 is part of the DGAT gene family (diacylglycerol acyltransferases) that codify key enzymes involved in the final step of triacylglycerol biosynthesis in various tissues and milk. In many cattle breeds the non-synonymous polymorphism K232A, from the 8th exon of the DGAT1 is a genetic marker, with major effects on milk yield and composition. In river buffaloes, the presence of the fixed K allele strongly indicates an increase in milk fat content as a result of selection. To date, only a few polymorphisms from the buffalo DGAT1 gene have been associated with milk composition. This study aims to test the associations between the synonymous polymorphism c.1053C>T from the 13th exon of the buffalo DGAT1 gene with milk qualitative parameters (fat percentage, protein percentage, and lactose content) in the Romanian buffalo breed. The properties of 200 milk samples were analysed using a mixed-effect model applied to longitudinal data spanning four seasons. Results indicated that the season had a significant impact ($p < 0.001$) on the fat and protein percentage, as well as lactose content in the milk. Additionally, a noteworthy association ($p < 0.001$) was observed between buffalo age and fat percentage. However, no significant association was found between genotype and milk quality.

Key words*: buffalo, DGAT1 gene, polymorphisms, milk composition, genetic associations.*

INTRODUCTION

Comparative analysis of the composition of buffalo's milk and cattle shows that the superior nutritional values of the milk and the dairy products of buffaloes are beneficial for human health (Contarini and Povolo, 2013; Khan et al., 2017, Marcone et al., 2017; Reddi et al., 2018). Compared to cow's milk, buffalo milk is richer in almost all milk nutrients (fat, lactose, protein, casein, ash and Ca, vitamins C and E) (Zicarelli, 2004; Abd El-Salam and El-Shibiny, 2011; Khan et al., 2019). Apart from being renowned for their mozzarella cheese production, buffaloes possess numerous other superior characteristics compared to cattle: meat composition is considerably lower in total fat content, including cholesterol (Naveena et Kiral, 2014; Guerrero-Legarreta et al., 2020), and the species' total methane production is lower

(Moss et al., 2000; Calabro et al., 2013; Khan et al., 2021). These properties, which match a perspective shift in consumers towards a healthier diet, have led the global buffalo population to increase at a steady rate of 14.7 ± 5.0% per annum, in the last 50 years (1968-2018). Buffalo milk production accounts for only 15.14% of global milk production. Europe contributes 2.8% to this market, but also with the highest added value obtained from Italian mozzarella. (Minervino et al., 2020). Advances in the molecular field have led to more efficient approaches in improving the genetic value of animals, using molecular information in breeding schemes. Many QTLs (Quantitative Trait Loci) with a major effect on milk yield and composition were first identified in the cattle genome, in the centromeric region of BTA14 that includes the DGAT1 gene, encoding the diacylglycerol acyltransferase1 (Dekkers, 2004; Bouwman et al., 2011). The K232A polymorphism inside the DGAT1 gene has been shown to be associated with milk composition and yield in various cattle breeds, like Holstein-Friesian, Fleckvieh, and Jersey (Grisart et al., 2002; Spelman et al., 2002; Winter et al., 2002; Kuhn et al., 2004; Ripoli et al., 2006). In several cattle breeds, the A allele was associated with milk yield, and the K allele with higher fat and protein content (Grisart et al., 2002; Winter et al., 2002; Spelman et al., 2002; Thaller et al., 2003; Weller et al., 2003; Gautier et al., 2007; Argov-Argaman et al., 2013). In buffaloes, the first studies on gene polymorphisms were performed using the cattle reference genome. Following the recent sequencing, re-sequencing, and annotation of the buffalo genome, numerous studies have subsequently concentrated on pinpointing genomic polymorphisms linked to significant economic traits (Yuan et al., 2007; Mishra et al., 2007; Shi et al., 2012; Cardoso et al., 2015; de Freitas et al., 2016; Silva et al., 2016; Khan et al., 2021). Initial investigations in buffalo have focused on the K232A polymorphism observed in cattle, due to the strong correlation of the DGAT1 polymorphism with milk FP in cattle (Conte et al., 2010). In contrast, research indicates that within water buffalo populations, the K allele appears to be consistently prevalent, owing to both natural and artificial selection processes spanning numerous generations (Tantia et al., 2006; Shi et al., 2012). This phenomenon provides an explanation for the elevated fat content observed in buffalo milk. DGAT (diacylglycerol acyltransferases) is a key enzyme group involved in the final steps of triacylglycerol biosynthesis in the adipose tissue, intestines, liver, and milk. Generally, these enzymes are controlled by a gene family DGAT (Yen et al., 2008), which includes several subfamilies, DGAT1 and DGAT2, DGAT3, and WAX-DGAT identified in different organisms (Turchetto-Zolet et al., 2016; Bhatt-Wessel et al., 2018). Comparative analysis of collinearity and gene synteny reveals a high conservation degree between buffalo and cattle genomes (Wang et al., 2021; Liu et al., 2020). Many other candidate genes for lipid metabolism have been identified in the buffalo genome by different authors, including A2M, DGAT1, GHRL, LEP, MC4R, PRL, SCD, SREBF1, STAT1, and TG. Another 4 genes (APOB, CTNND2, FHIT, and ESSRG) were identified as candidates in Genome-Wide Association Studies (GWAS) (Camargo et al., 2015; Du et al., 2019). GWAS is considered the golden standard in genomic selection technology. Currently, few GWAS exist on river buffaloes (*Bubalus bubalis*), and few or no associations of the DGAT1 gene polymorphisms with milk yield and composition were established (Liu et al., 2020). The most important polymorphisms associated with milk yield and composition were identified in the 5' UTR region and the 13th and 17th exons of the DGAT1 gene (Yuan et al., 2007; Cardoso et al., 2015; Li et al., 2018), while other SNPs were found in various buffalo breeds, but no associations were studied (Mishra et al., 2007; Yuan et al., 2007; Raut et al., 2012; Silva et al., 2016; Gu et al., 2019). For this reason, the main goal of this research is to test the associations of the DGAT1 polymorphism with lactogenic parameters. The DGAT1 gene polymorphism, specifically c.1053C>T, was detected through transcript analysis of the DGAT1 gene in Italian Mediterranean and Romanian buffalo breeds (Gu et al., 2019). Thus, the scope of this study is to test the associations between this polymorphism and fat percentage (FP), protein percentage (PP) and milk lactose content, in a cohort of 50 female Romanian buffaloes, from the Research and Development Station for Buffalo Breeding Sercaia (Brasov County).

MATERIALS AND METHODS

Biological material

The formation of the Romanian buffalo breed took place in the early 1960s through the introgression of the European river buffalo *(Bubalus bubalis*) with the Bulgarian Murrah breed. Today over 90% of the buffalo livestock is raised on small farms from Transylvania (Brașov, Cluj, Sibiu, and Mureș counties) (Matiuti et al., 2020). Two different populations, with distinct genetic backgrounds were identified by Noce et al (2021) in genetic diversity studies (90k SNPs): the Mera buffalo population in Cluj County, distributed among small farms, with a genetic background admixture from Italian-Mediterranean, Hungarian, and Bulgarian origins; and the animals from the Research and Development Station for Buffalo Breeding Sercaia (Brasov County), which have a defined genotype, due to intense selection programs and environmental adaptation to the cold climate.

In the present study, we proposed to analyse this population of Sercaia Station, to test the associations between c.1053C>T polymorphism, from the 13th exon of DGAT1 gene, and milk production characteristics. The animals from this facility are in the official production control program.

Blood and milk samples collection

For the gene polymorphisms study and associations procedures with milk qualitative parameters, we used blood samples collected from 50 buffalo females, randomly selected from the Research and Development Station for Buffalo Breeding Sercaia, Brasov County (45o50'01.4" N and 23o27'07.1"). The animals, in different lactation phases, were fed identically during the sampling period. From these selected females, 200 individual morning milk samples (50 ml/sample and 50 sample/season) were collected in four successive seasons (autumn, winter, spring and summer). Vacutainers with K2EDTA were used to collect blood samples, which were immediately refrigerated (4ºC ± 1ºC) and transported to the laboratory for DNA extraction. The same procedure was applied for milk samples, which were collected in 100 ml Falcon tubes (50 ml/sample). Throughout the sampling process, particular attention was paid to animal welfare.

In silico analysis of the c.1053C>T polymorphism of the DGAT1 gene

In this study we aimed to identify the c.1053C>T polymorphism from the 13th exon, in a 483 bp DNA fragment of the buffalo DGAT1 gene. In the buffalo genome, this gene is located on the 15th (BBU15) chromosome, has 17 exons and a total length of ~8,8 kb (NCBI Gene ID 102390126). The sequence of interest corresponds to the genomic positions g.81,363,550 - g.81,364,033 in the NCBI genomic sequence NC_059171.1/NCBI (GCF_019923935.1/NDDB_SH_1 *Bubalus bubalus*, Murrah breed).

The sequence of interest was amplified by PCR with a specific primers pair (forward 5'-ATGGGCGACCGCGGCGG-3' and reverse 5' – GGAGCATGGGCTTGTAGA-3' (Gu et al., 2017) corresponding to the region between the 12th and 15th exons on the GenBank DGAT1 gene sequence DQ886485.1 of water buffalo. The two sequences produced a perfect alignment in the Blast analysis, and the position of the SNP mutation, recognized by *Dde*I restriction enzyme, is highlighted in Figure 1. The C>T mutation, which falls at the genomic position g.81,363,844 corresponds to the mutation position - c.1053C>T in the NCBI sequence DQ120929.1 *Bubalus bubalis* diacylglycerol O-acyltransferase 1 (DGAT1) mRNA, complete cds. The C>T is a synonymous mutation that does not modify the amino acid sequence ($Y^{Ala/Ala}$) specified by the two codons.

DNA extraction

The DNA samples were obtained from 200 µl blood, collected on vacuum tubes with K2EDTA, with Quick DNA Microprep Plus Kit, following the manufacturer's instructions (BioZyme, St Joseph, Missouri, USA). All samples were analysed to determine the DNA concentration and purity on Spectrophotometer NanoDrop ND1000, and range between 1.8-2 purity (optimum), and a quantity between 50 and 180 ng/µl.

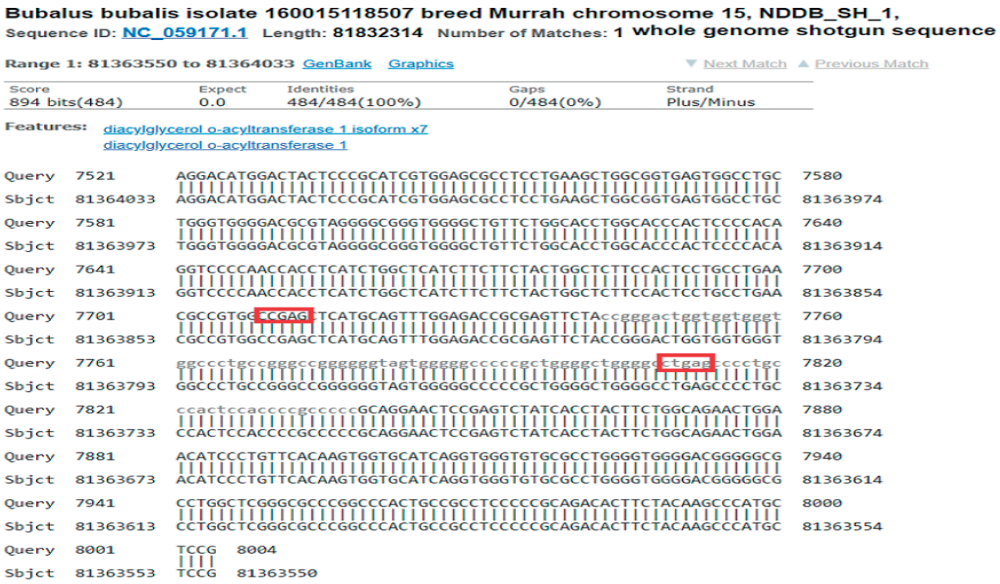


Figure 1. Blast analysis of the genomic sequence NC_059171.1 (g. 81,363,550 - g.81,364,033) and DGAT1 gene from GenBank (DQ886485.1), amplified by PCR (exons: 12-15) and the position of the *Dde*I restriction enzyme sites with potential mutation C>T (DQ886485.1-7710)

Genetic analysis

A final volume of 25 µl PCR mix was set up for each sample with 5x FirePol Master mix (BioZyme) – 5 µl; 1 µl of each primer (forward and reverse) (10 pmol/µl), DNA template – 2 µl (100 ng) and molecular grade water -16 µl. The following conditions were used for the PCR assay: 97ºC for 4 min; followed by 35 cycles at 97ºC (45 s), 63.2ºC (45 s), and 72ºC (45 s); and a final extension step at 72ºC for 8 min, maintaining 4ºC thereafter, in an Eppendorf MasterCycler (Eppendorf, Germany). All amplimers, of 483 pb, were subjected to digestion with the *Dde*I restriction enzyme. The enzymatic digestion mixture includes 10 µl PCR product, 5U of *Dde*I endonuclease (New England, Biolab), and 2.5 µl of the enzyme's buffer. For digestion, an overnight incubation at 37 ºC was performed. The digestion products were analysed in agarose gel (2.5%) electrophoresis, with 1x TBE buffer, and stained with RedSafe (Intron Biotechnology). The 483 DNA fragment amplified by PCR can be cleaved by the *Dde*I restriction enzyme in two positions, that correspond to TT genotypes, with two visible bands on the agarose gel, of 189/194 bp and 100 bp. After digestion, the C allele also presents two fragments but with different lengths of 289 bp and 194 bp. The heterozygous (CT) genotype can be distinguished in the agarose gel through a profile of 289 bp, 189/194 bp, and 100 bp.

Milk quality parameters analysis

In each season (autumn, winter, spring, summer) 50 milk samples (50 ml/sample) collected from buffalo females were analysed on an Ultrasonic milk analyser (Lactoscan MCCW/UASVM of Cluj-Napoca). The working method aimed to determine milk quality parameters (fat % (FP), protein % (PP), and lactose content).

Statistical analysis

Allelic and genotype frequencies were determined by simple counting. Possible deviations from Hardy-Weinberg equilibrium (HWE) were tested by chi-squared test, using the HWE.chisq function call from the genetics package (1.3.8.1.3) in R (Table 1).

The association analysis between the SNP locus and milk traits was performed using a linear mixed-effects model due to the nested structure of the longitudinal data (Bates et al., 2015). Apart from the genotype, two other variables that may also influence milk production were inserted in the model as fixed effects, the season (categorical with four levels), and the age group (categorical with three levels, discretizing the

buffalo age in ranges [4, 7], [7, 10], and [10, 20] years). The milk samples were collected over the course of seasons, with multiple observations per buffalo. Because of that, milk properties for the individual buffaloes are correlated throughout seasons, hence the necessity to introduce nested random effects (intercepts) for each buffalo into the model.
The model formula is the following:

$$y_{hjkl} = \beta_0 + \beta_1 \times GENOTYPE_h + \beta_2 \times SEASON_j + \beta_3 \times AGE_{GROUP_k} + ID_l + \epsilon_{hjkl}$$

Where: Y_{hjkl} represents the modeled phenotype of the milk (FP, PP, lactose) for each buffalo ID_l in season $SEASON_j$ and AGE_{GROUPk}; β_0 is the overall mean of the measured phenotype; β_1, β_2, β_3 are the regression coefficients of the genotype, season and age group classes respectively; ϵ_{hjkl} represents the residual error. $GENOTYPE_h$, $SEASON_j$ and AGE_{GROUPk} are set as the fixed effects of the model and the ID_l of each buffalo as the random effect. The model assumptions for multicollinearity were tested using Variance Inflation Factors (VIFs). For the lactose and protein percentage residuals, normality tests including the Shapiro-Wilk, Anderson-Darling, and Lilliefors (Kolmogorov-Smirnov) indicated no significant departure from normality, with p-values well above the 0.05 threshold. However, for fat percentage residuals, these normality tests failed to meet the assumption of normality. Consequently, a sensitivity analysis was conducted, to evaluate the model's robustness across each 25% segment of quantiles, to ensure the model's reliability despite the non-normality observed in the fat percentage residuals. All analyses were performed using R (R Core Team 2023). The restricted maximum likelihood (REML) estimates of the parameters in the linear mixed-effects models were determined by using lmer function in the lme4 package (1.1.33). Additionally, ggplot2 package (3.4.2) was used for data visualization.

RESULTS AND DISCUSSIONS

From the PCR amplimers digested with *Dde*I enzyme, all possible genotypes, CC, CT, and TT (Figure 2) have been identified in the analysed population. The results of allelic and genotype frequencies and the departure from HWE can be viewed in Table 1.

Table 1. Allelic and genotype frequencies and the departure from Hardy-Weinberg equilibrium

Genotype (SNP c.1053 C>T) frequency			No of animals	Allele frequency		HWE
CC	CT	TT		C	T	χ^2=0.0868
0.26	0.52	0.22	N=50	0.52	0.48	*P*=0.7835

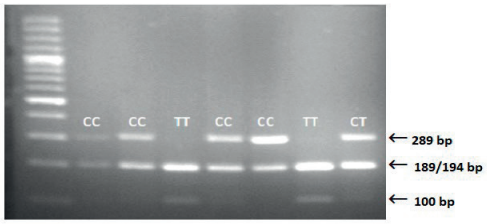


Figure 2. Genotypes of DGAT1 gene (c.1053C>T polymorphism) in Romanian buffalo breed. Line 1:100 bp step ladder (Promega, USA), lines 2-8: CC genotypes with 289 bp and 194 bp bands, TT genotypes with 189/194 bp and 100 bp bands, and CT genotypes with 289, 189/194 and 100 bp bands

The results of the gene polymorphism study in the Romanian buffalo breed, regarding the C>T mutation (c.1053) in exon 13 of the DGAT1 gene, are consistent with those reported by Gu et al. (2019) in studies on differential gene expression. They indicate a higher frequency of the C allele (0.52) and a lower frequency of the T allele (0.48) in the Romanian buffalo compared to the Italian Mediterranean breed, in which the C allele was determined in a lower proportion (C=0.46). To the best of our knowledge, currently, the synonymous mutation c.1053 C>T has only been identified in these two breeds. Querying the relevant literature on this mutation shows the presence of only the T allele at this locus in the Chinese buffalo breed (Yuan et al., 2007), while in various other water buffalo breeds (Bhadawari, Mehsana, Murrah, and Surti buffaloes), including Indian Murrah, studies show the presence of only the C allele (Mishra et al., 2007; Venkatachalapathy et al., 2008). The descriptive statistics of the milk composition parameters are presented in Table 2.

Table 2. Descriptive statistics of the milk composition parameters in Romanian Buffalo breed

Trait	Season	N	Mean ± s_x	SD	Minimum	Maximum	CV%
FP	Autumn	50	8.015 ± 0.027	0.188	7.322	8.501	2.35
	Winter	44	7.974 ± 0.057	0.377	7.111	8.719	4.73
	Spring	49	7.945± 0.045	0.317	7.308	8.487	3.99
	Summer	50	7.735 ± 0.022	0.154	7.466	8.224	1.99
	All	193	7.916 ± 0.021	0.290	7.111	8.719	3.66
PP	Autumn	50	4.028 ± 0.013	0.090	3.912	4.347	2.24
	Winter	44	4.093 ± 0.032	0.213	3.809	4.592	5.21
	Spring	49	3.964 ± 0.027	0.187	3.702	4.391	4.71
	Summer	50	4.353 ± 0.023	0.164	3.999	4.610	3.77
	All	193	4.111 ± 0.016	0.225	3.702	4.610	5.46
Lactose	Autumn	50	4.138 ± 0.022	0.162	3.738	4.453	3.93
	Winter	44	3.896 ± 0.016	0.109	3.716	4.113	2.81
	Spring	49	3.930 ± 0.028	0.197	3.605	4.466	5.01
	Summer	50	3.813 ± 0.023	0.164	3.524	4.230	4.31
	All	193	3.946 ± 0.015	0.202	3.524	4.466	5.12

N = number of samples, s_x = standard error; SD = standard deviation, CV = coefficient of variation, Fat Percentage FP, Protein Percentage PP

Regarding fat percentage and protein percentage, the minimums were observed in winter (7.11% FP) and spring (3.70% PP), while for lactose the lowest value was found in summer (3.52 lactose content). Furthermore, the winter and spring seasons generally show the highest variances, with a maximum standard deviation of 0.37% FP and 0.21% PP in winter, and a variance of ~0.2 in lactose content during spring.

Association Study

Tables 3, 4, and 5 present the estimates of the linear mixed effect models on the association between genotype, season, and age groups. For all mixed effect models, the reference group was chosen among the buffalos between 4 and 7 years old, having the CC genotype, and for the measurements taken in the Autumn season. The CC genotype represents the wild type allele, while the age group and season were the first groups among the observations. All found differences are present between these reference groups and others. Moreover, in the case of the genotype, we are interested in whether any polymorphism is associated with a change in the milk quality.

The overall mean fat percentage in milk samples was estimated to be 7.967% (95% confidence interval (CI): 7.861%, 8.073%) at the reference level (for genotype CC, in Autumn for buffaloes in the age group [4,7] years). In the case of protein percentage, the average was found to be 4.046% (95% CI: 3.975%, 4.117%), while the mean lactose content of the milk was 4.150 (CI: 4.085, 4.216) within the reference group.

To interpret the fixed effects of the model, the estimated coefficients can be used. For instance, for milk samples taken in summer from buffaloes in the same age group, we observe an increase in protein percentage of +0.325% (CI: +0.262%, +0.389%) compared to the reference level.

Table 3. Coefficient estimates β of the linear mixed-effect models for fat percentage of the milk samples

Mixed Effect Model of Fat Percentage (FP)				
Independent Variables		Estimated Coefficients β	Lower 95% CI	Upper 95% CI
INTERCEPT		7.967	7.861	8.073
GENOTYPE	CC	Ref.		
	CT	0.020	-0.070	0.109
	TT	0.071	-0.034	0.176
SEASON	Autumn	Ref.		
	Winter	-0.043***	-0.148	0.061
	Spring	-0.070***	-0.172	0.031
	Summer	-0.280***	-0.381	-0.179
AGE GROUP	[4, 7]	Ref.		
	(7, 10]	-0.053***	-0.143	0.035
	(10, 20]	0.123***	0.035	0.212

95% CI = Confidence Interval; *p < 0.05, **p < 0.01, ***p < 0.001; Ref. stands for reference in the analysis.

Table 4. Coefficient estimates of the linear mixed-effect models for protein percentage of the milk samples

Mixed Effect Model of Protein Percentage (PP)				
Independent Variables		Estimated Coefficients β	Lower 95% CI	Upper 95% CI
INTERCEPT		4.046	3.975	4.117
GENOTYPE	CC	Ref.		
	CT	-0.028	-0.090	0.034
	TT	-0.057	-0.130	0.016
SEASON	Autumn	Ref.		
	Winter	0.068***	0.002	0.134
	Spring	-0.064***	-0.128	0.000
	Summer	0.325***	0.262	0.389
AGE GROUP	[4, 7]	Ref.		
	(7, 10]	0.003	-0.059	0.065
	(10, 20]	0.026	-0.036	0.087

95% CI = Confidence Interval; *p < 0.05, **p < 0.01, ***p < 0.001; Ref. stands for reference in the analysis

The mixed effects model reveals a notable association ($p < 0.001$) between buffalo age groups and seasons concerning FP as seen in Table 3. Likewise, both the PP and lactose models exhibit a significant ($p < 0.001$) association with seasons (Tables 4 and 5).

The combined effect of milk parameters for different genotype groups and seasons can be seen in Figure 3. Notably, the results reveal the absence of influences of the genotypes on milk parameters. A possible source of statistical ambiguity is the limited number of samples available. Specifically, we note that all of the outliers excluded from the analysis are found in Winter and Spring, as these seasons have overall higher variability between individuals. We posit that as a synonymous mutation, further studies on this mutation and testing for linkage disequilibrium between loci, could provide additional information about this polymorphism.

Insights from Research on the Buffalo Genome

The limited number of association studies on gene polymorphisms and productive traits in the water buffalo is primarily explained by the later appearance of the *Bubalus bubalis* genomic reference (Williams, 2017) compared to the *Bos taurus* genome. Thus, this study aims to complement the research on the possible influences of gene mutations on the expression of milk productive parameters such as fat percentage (FP), protein percentage (PP), and lactose.

Table 5. Coefficient estimates of the linear mixed-effect models for the lactose in the milk samples

Mixed Effect Model of Lactose				
Independent Variables		Estimated Coefficients β	Lower 95% CI	Upper 95% CI
INTERCEPT		4.150	4.085	4.216
GENOTYPE	CC	Ref.		
	CT	0.025	-0.031	0.080
	TT	-0.003	-0.068	0.062
SEASON	Autumn	Ref.		
	Winter	-0.241***	-0.306	-0.177
	Spring	-0.207***	-0.270	-0.144
	Summer	-0.325***	-0.387	-0.262
AGE GROUP	[4, 7]	Ref.		
	(7, 10]	-0.036	-0.092	0.019
	(10, 20]	-0.041	-0.096	0.014

95% CI = Confidence Interval; *p < 0.05, **p < 0.01, ***p < 0.001; Ref. stands for reference in the analysis

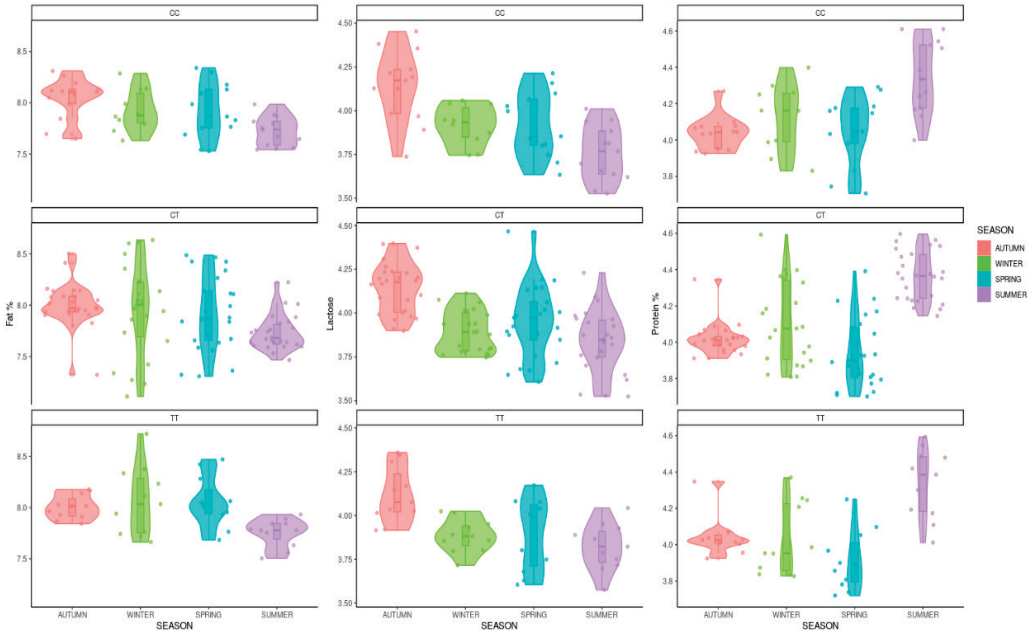


Figure 3. Fat Percentage, Lactose and Protein Percentage for different genotype groups and seasons. The violin plot combines the probability density and box plots, while individual milk sample measurements are visible as dots

A priori hypotheses about the effects of synonymous mutations in genes, which can affect gene expression through various molecular processes such as splicing, mRNA stability, folding, and translation (Wang et al., 2021), have led to the identification of several synonymous mutations associated with productive traits, including the DGAT1 gene (Cardoso et al., 2015). Certain synonymous mutations in genes, although they do not change the specified amino acid in the protein, can be in linkage disequilibrium with other SNPs in the genome, affecting the expression of complex traits (Bouwman et al., 2011).

The role of the DGAT1 gene in the biosynthesis and storage of milk fat has been demonstrated in double-knockout lactation-deficient mice (Smith et al., 2000). The mutations that can have substantial effects on the differentiated expression of genes affect the regulatory regions of the genes (promoters, enhancers, silencers, and insulators). Among the studies identifying mutations in the DGAT1 gene, the VNTR polymorphism in the 5'UTR region of the DGAT1 gene (Cardoso et al., 2015) is notable, accounting for 32% of the additive genetic variance (VA) of milk fat percentage (FP) in the Murrah buffalo breed.

Other studies of SNP associations in buffalo have been tested in specific breeds and need to be confirmed in association studies for each breed individually (Tantia et al., 2006; Mishra et al., 2007; Raut et al., 2012; Venkatachalapathy et al., 2013; Cardoso et al., 2015; de Freitas et al., 2016). Thus, significant associations ($P<0.05$) have been identified between the nonsynonymous polymorphism g.11785 T> C in exon 17, which changes the amino acids Ala484Val and the milk protein percentage (PP) and fat percentage (FP) in the Murrah buffalo breed, and in the Italian buffalo breed (De Freitas et al., 2016; Li et al., 2018). In the river buffalo, an SNP polymorphism (g.8330T>C) in exon 13 of the gene appears to be associated with the protein and fat percentage in milk (Li et al., 2020). Several other studies identified different SNP polymorphisms in intron 1, exons 7-9, and intron 7 of the DGAT1 gene from different buffalo breeds, but many without association studies (Mishra et al., 2007; Yuan et al., 2007; Raut et al., 2012; Silva et al., 2016).

GWAS, despite being a standard in genomic selection, face limitations due to the reliance on the number of allelic variants on microchips. The lead SNP with the tightest association p-value may not always be the causal SNP. This

necessitates considering numerous other SNPs in linkage disequilibrium as potentially causal (Chatterjee and Ahituv, 2017). A comprehensive GWAS, investigating the DGAT1 gene's impact on milk's fatty acid composition was conducted by Cruz et al. in 2019. The authors utilized various statistical methods to elucidate its correlation with milk composition variability. This study established the DGAT1 gene as a key determinant in the variation of milk's fatty acid profile, in conjunction with two other genes, PLBD1 and MGST1, particularly when DGAT1 was included as a fixed variable in the analysis. The exclusion of DGAT1 as a fixed covariate led to the discovery of numerous SNPs. Therefore, the effects of genomic polymorphisms should be cautiously analysed to identify optimal associations with quantitative traits.

Additionally, Liu et al. (2020) linked polymorphisms in the DGAT2, DGAT2L7, and DGAT2Ln genes to the quantity of milk fat in river buffalo through GWAS explaining for the extended role of the genes in the DGAT family. Further genetic studies have pinpointed specific polymorphisms in a haplotype of the DGAT1 gene that are associated with both, milk protein and fat percentages (Liu et al., 2020).

CONCLUSIONS

The composition and fat content of milk are crucial factors in the dairy sector, influencing both the economic success of farms and the consumer preference for high nutritional value dairy products. Notably, buffalo milk is valued for its lower levels of saturated fatty acids and cholesterol, making it an alternative dietary choice and a key point of breeding programs aimed at enhancing milk yield and quality.

In the context of the population examined in this research, we suggest that future studies should aim to sequence the entire exon in a broader sample group. Considering the role of synonymous polymorphism, additional research on this specific mutation, along with analyses of linkage disequilibrium among loci, could yield deeper insights into the nature and implications of this genetic variation.

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ESTIMATION THE GENETIC PARAMETERS FOR BIRTH WEIGHT AND WEANING WEIGHT IN LIMOUSINE BREED

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Abstract

The objective of this study was to estimate the breeding values and genetic parameters for birth weight and weaning weight in Limousine cattle breed with multivariate maternal animal model for selection. Data consisted of records of 1207 cattle from Romanian Breeding Association for Beef cattle. The direct breeding values for birth weight were between -7.890 and 7.049 and for weaning weight -55.381 and 60.818 kg. The maternal breeding values for birth weight ranged between -1.701 and 1.810 and for weaning weight -22.453 and 20.747. The direct and maternal heritability for birth weight were 0.105, respectively 0.035, for weaning weight 0.662, respectively 0.246. The total heritability for birth weight was 0.92 and 0.655 for weaning weight.

Key words*: birth and weaning weight, breeding values, genetic parameters, multivariate maternal animal model.*

INTRODUCTION

Beef farming has an important role in agriculture in many countries. The factors which influence the meat production are genetic factors: breed, individual, physiological factors as age, sex and environmental factors. In beef cattle the phenotype of calves is influenced by the ability of dam to assure favourable nutritional environmental for progeny (Grosu & Oltenacu, 2005). The birth weight and weaning weight are the traits influenced by maternal genetic effects. The maternal animal model is a good model for genetic evaluation of growth traits because it presents both the genetic and maternal effects. The animals are selected in function of the direct breeding values and maternal breeding values. In the breeding program of Limousine breed are included the meat production traits and reproduction traits. The multi-trait animal model is the optimal model for genetic evaluation of cows because it uses the performance of cows and its relatives, the genotypic and environmental correlation between the traits and the relative economic values of the traits including in breeding objective (Grosu et al., 2019). The use of this model leads to an increase in the accuracy of the selection. The simultaneous genetic evaluation for multiple traits can be noticed worldwide, because this makes it possible the use the information supplied by the genetic correlations between the traits. Determines a higher accuracy of the estimated breeding values for the traits with low heritability (Grosu et al., 2013). The multiple trait model was described by Henderson & Quass (1976), Pollak & Quass (1981), Polak et al. (1984). Multi-trait animal model can be used for calving and reproduction, for disease traits, for production traits and conformation traits in evaluation of cattle, in beef breeds for the meat and reproduction performance, calf survival and growth traits. Weik et al. (2022) reported that in the breeding programs can incorporate both maternal and finishing traits as selection criteria. The objective of this study was to estimate the breeding values and genetic parameters for birth weight and weaning weight in Limousine cattle breed with multivariate maternal animal model for selection.

MATERIALS AND METHODS

The data used in this study provide from Romanian Breeding Association for Beef cattle. For Limousine breed, the pedigree contents 2562 animals: 1207 cattle with records, 149 sire

and 1206 dams. The cattle with records from Limousine were born in 2022. The values for calves' birth weight and weaning weight were checking with Grubs test if the values were in acceptable limits.

$$\hat{v} = \frac{X_{MIN,MAX} - \bar{X}}{\sigma}$$, where:

$\hat{v} = the\ tested\ value$;

X= the value tested;

σ = standard deviation;

$\bar{X}$ = the mean.

The fixed part of the model included the sex of calves with two levels, male and female. The random effects were the direct genetic effects, the maternal genetic effects and the permanent environmental effects. For estimate the genetic parameters was used the restricted maximum likelihood method with a multivariate maternal animal model. Statistical analysis was performed using R 3.5.1. statistical software (https://www.r-project.org/).

The multivariate maternal model was (Mrode & Thompson, 2005):

$$y = Xb+Zu+Wm+Spe+e$$

y = the vector of observations,

$$y = \begin{bmatrix} y1 \\ y2 \end{bmatrix}$$

$$X = \begin{bmatrix} X_1 & 0 \\ 0 & X_2 \end{bmatrix}, \hat{b} = \begin{bmatrix} \hat{b}_1 \\ \hat{b}_2 \end{bmatrix}, Z = \begin{bmatrix} Z_1 & 0 \\ 0 & Z_2 \end{bmatrix},$$

$$\hat{u} = \begin{bmatrix} \hat{u}_1 \\ \hat{u}_2 \end{bmatrix} \quad W = \begin{bmatrix} W1 & 0 \\ 0 & W2 \end{bmatrix}$$

$$S = \begin{bmatrix} S1 & 0 \\ 0 & S2 \end{bmatrix} \; p = \begin{bmatrix} pe1 \\ pe2 \end{bmatrix} e = \begin{bmatrix} e1 \\ e2 \end{bmatrix}$$

$$\begin{bmatrix} y_1 \\ y_2 \end{bmatrix} = \begin{bmatrix} X_1 & 0 \\ 0 & X_2 \end{bmatrix} \begin{bmatrix} b_1 \\ b_2 \end{bmatrix} + \begin{bmatrix} Z_1 & 0 \\ 0 & Z_2 \end{bmatrix} \begin{bmatrix} u_1 \\ u_2 \end{bmatrix} + \begin{bmatrix} W_1 & 0 \\ 0 & W_2 \end{bmatrix} \begin{bmatrix} m_1 \\ m_2 \end{bmatrix} + \begin{bmatrix} S_1 & 0 \\ 0 & S_2 \end{bmatrix} \begin{bmatrix} pe_1 \\ pe_2 \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \end{bmatrix}$$

y1 = vector of observations for the trait 1;

y2 = vector of observation for the trait 2;

b = the vector of the fixed effect represented by sex;

b1 = the vector of observation of the fixed effect represented by sex for trait 1;

b2 = the vector of observation of the fixed effect represented by sex for trait 2;

u = the vector of the random animal effects;

u_1= the vector of the random animal effects for trait 1; u_2 =the vector of the random animal effects for trait 1;

m= the vector of the random maternal genetic effects;

m_1= the vector of the random maternal genetic effects for trait 1; m_2= the vector of the random maternal genetic effects for trait 2;

p_e = the vector of the permanent environmental effects;

p_{e1}= the vector of the permanent environmental effects for trait 1; p_{e2}= the vector of the permanent environmental effects for trait 2;

e = the vector of the random residual effects;

e_1= the vector of the random residual effects for trait 1; e_2= the vector of the random residual effects for trait 2;

X, Z, W and S are the incidence matrices referring to animal performance, to the fixed effects, to the direct effects, the maternal effects and to the permanent environmental effects.

$$var \begin{bmatrix} u_1 \\ u_2 \\ m_1 \\ m_2 \\ pe_1 \\ pe_2 \\ e_1 \\ e_2 \end{bmatrix} \begin{bmatrix} g_{11}A & g_{12}A & g_{13}A & g_{14}A & 0 & 0 & 0 & 0 \\ g_{21}A & g_{22}A & g_{23}A & g_{24}A & 0 & 0 & 0 & 0 \\ g_{31}A & g_{32}A & g_{33}A & g_{34}A & 0 & 0 & 0 & 0 \\ g_{41}A & g_{42}A & g_{43}A & g_{44}A & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & q_{11} & q_{12} & 0 & 0 \\ 0 & 0 & 0 & 0 & q_{21} & q_{22} & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & r_{11} & r_{12} \\ 0 & 0 & 0 & 0 & 0 & 0 & r_{21} & r_{22} \end{bmatrix}$$

A = the kinship matrix between animals;

I = the identity matrix;

σ_a^2= the additive genetic variance for the direct effects;

σ_m^2 = the additive genetic variance for the maternal effects;

σ_{am} = the additive genetic covariance between the direct and maternal effects;

σ_{pe}^2 = the variance due the permanent environmental effects;

σ_e^2 = the variance of the residual error.

- the direct heritability $h_{a=}^2 \sigma_a^2/\sigma_p^2$

where σ_p^2 is the phenotypic variance

- the maternal heritability = $h_{m=}^2 \sigma_m^2/\sigma_p^2$
- the covariance between direct and maternal effects as proportion of the phenotypic variance ($\sigma_{am}/\ \sigma_p^2$)
- the total heritability (Wilham et al., 1972):

$$h_T^2 = \frac{\sigma_a^2 + 0.5\sigma_m^2 + 1.5\sigma_{am}}{\sigma_p^2}$$

h_T^2 is the total heritability, and σ_p^2 is the phenotypic variance

The standard error of heritability was:

$$s.e.(h^2 = \sqrt{\left[\left(\frac{\partial h^2}{\partial \sigma_g^2}\right)^2 (se_g)^2 + \left(\frac{\partial h^2}{\partial \sigma_e^2}\right)^2 (se_e)^2 + 2\left(\frac{\partial h^2}{\partial \sigma_g^2}\right)^2 \left(\frac{\partial h^2}{\partial \sigma_e^2}\right)^2 \rho_{g,e} se_g se_e\right]}$$

where:

$$\frac{\partial h^2}{\partial \sigma_g^2} = \frac{\sigma_e^2}{(\sigma_g^2+\sigma_e^2)^2}$$

$$\frac{\partial h^2}{\partial \sigma_e^2} = \frac{-\sigma_g^2}{(\sigma_g^2+\sigma_e^2)^2}$$

(Hoj Edwards, 2017).

- the ratio of the maternal permanent environment to phenotypic variance (c^2);
- r_{am} the genetic correlation between the direct and maternal effects:

$$r_{am} = \frac{\sigma_{am}}{\sqrt{\sigma_a^2 \cdot \sigma_m^2}}$$

$$G_1 = \begin{bmatrix} g_{11} & g_{12} \\ g_{21} & g_{22} \end{bmatrix}$$

$$G_2 = \begin{bmatrix} g_{13} & g_{14} \\ g_{23} & g_{24} \end{bmatrix}$$

$$G_3 = \begin{bmatrix} g_{33} & g_{34} \\ g_{43} & g_{44} \end{bmatrix}$$

G = the matrix for direct and maternal effects

$$k1 = G_1 * A^{-1}$$

$$k2 = G_2 * A^{-1}$$

$$k3 = G_3 * A^{-1}$$

g_{11}, g_{22} = the additive genetic variance for the direct effects for traits 1, 2;

g_{13}, g_{14}= the additive genetic covariance between the direct and maternal effects;

g_{33}, g_{44}= the maternal genetic variance:

$$Q = \begin{bmatrix} q_{11} & q_{12} \\ q_{21} & q_{22} \end{bmatrix} \quad R = \begin{bmatrix} r_{11} & r_{12} \\ r_{21} & r_{22} \end{bmatrix}$$

Q = matrix for permanent environmental effects ;

q_{11}, q_{22} = the variance for permanent environmental effects for traits 1, respectively 2;

q_{12}, q_{21} = the covariance for permanent environmental effects;

R = the matrix for residual effects;

r_{11}, r_{22}=the variance for residual effects

r_{12}, r_{21}=the covariance for residual effects.

RESULTS AND DISCUSSIONS

The growth traits are important economic traits in Limousine breed. In the Table 1 were presented the statistics for birth weight and weaning weight in Limousine breed.

The means for birth weight and weaning weight obtained in our study were influenced by the sex of animal. The weight at birth and weaning were greater for males than the females. In our study the number of females was greater than the number of males. The weight can be influenced by the different environmental conditions in the farms.

The coefficient of variability was smaller for birth weight showing the population present homogeneity for this trait, for weaning weight the coefficient of variability was higher showing the heterogeneity for this trait. Putra et al. (2020) reported the mean for Limousine males for birth weight was 38 kg and for weaning weight was 155.17 kg. The vales from our study for birth weight were higher than the values reported by Pilarczyk & Wojcik (2007) for females 31.2 and for males 33.5 kg but for the weight at 210 days the means were lower in our study than 237.9 for heifers and 256.2 kg for bulls. Jakubec et al. (2003) reported a mean for birth weight 29.19 kg and for the weight at 210 days of 216 kg in Limousine breed from Czech Republic, the values from our study were higher. Cundiff et al. (1986) reported the mean for birth weight 38.9 kg and for weight at 200 days 198 kg in Limousine breed. Rezende et al. (2022) reported a mean of 247.73 kg the weight at 210 days.

Table 1. The descriptive statistics for birth weight and weaning weight

Specification	Birth weight	Weaning weight
Mean±Standard error (kg)	38.111±0.130	222.502±1.037
Standard deviation	4.55	36.059
Coefficient of variability)%)	11.93	16.206
Number of cattle (head)	1207	1207
Mean±Standard error for males	39.463±0.288	231.202±2.179
Number of males (head)	302	302
Standard deviation	5.017	37.879
Coefficient of variability (%)	12.713	16.383
Mean±Standard error (kg) for females	37.66±0.144	219.6±1.604
Standard deviation	4.335	48.280
Coefficient of variability (%)	11.511	21.985
Number of females (head)	905	905

In the Table 2 were presented the means for birth weight and weaning weight for males and females obtained from BLUP solutions. The means from BLUP were realized a reciprocal correction of presented effects in model. The standard errors associated with the results were computed from the square root of the diagonal of the generalized inverse (Mrode & Thompson, 2005). The standard errors of means were lower from means estimated with BLUP methodology than the standard errors of arithmetic means. By Student test observed that were very significant difference between means for males and females for birth weight t calc>$t_{\infty;0.001}$, 5.22>3.29 and for weaning weight t calc>t∞;0.001, 3.83>3.290.

Table 2. The means for birth weight and weaning weight from BLUP solutions

Specification	Birth weight	Weaning weight
Mean±Standard error for males	39.433±0.171	230.444±1.256
Mean±Standard error (kg) for females	37.751±0.123	220.078±0.927

The direct and maternal breeding values for the birth weight and weaning weight for the best cattle in Limousine breed were presented in the Table 3.

Table 3. The direct and maternal breeding values for birth weight and weaning weight for the best cattle

Birth weight	Birth weight	Weaning weight	Weaning weight
Direct breeding values	Maternal breeding values	Direct breeding values	Maternal breeding values
7.049	1.810	60.818	20.747
7.007	1.789	60.020	20.605
6.844	1.684	60.013	20.175
6.819	1.683	59.863	19.855
6.819	1.673	58.218	19.218
6.736	1.645	57.939	19.157
6.715	1.607	57.767	18.762
6.709	1.603	56.974	18.717
6.684	1.580	56.877	18.696
6.677	1.563	56.541	18.497

The direct breeding values for the best cattle for birth weight ranged between 6.677 and 7.049 and the maternal breeding values were from 1.563 and 1.810. For weaning weight, the direct breeding values ranged between 56.541 and 60.818 and maternal breeding values were from 18. 497 and 20.747.

In the Table 4 were presented the relative direct breeding values for cattle for birth weight and weaning weight.

Table 4. The relative breeding values for birth weight and weaning weight

Birth weight	Weaning weight
Direct relative breeding values	Direct relative breeding values
134.420	134.855
134.212	134.398
133.419	134.394
133.299	134.308
133.299	133.366
132.897	133.207
132.792	133.109
132.763	132.654
132.644	132.599
132.608	132.407

In the Table 5 were presented the genetic parameters for birth weight and weaning weight estimated with multivariate maternal animal model.

Table 5. The genetic parameters for birth weight and weaning weight estimated with multivariate maternal animal model

Item	Birth weight	Weaning weight
σ_a^2	5.969	559.270
σ_m^2	2.012	207.960
σ_{am}	-1.122	-73.583
σ_{pe}^2	46.73	1.598
σ_e^2	1.103	1.651
σ_p^2	56.944	844.064
c^2	0.820	0.002
σ_{am}/σ_p^2	-0.019	-0.087
r_{am}	-0.323	-0.215
h_a^2	0.105±0.082	0.662±0.019
h_m^2	0.035±0.105	0.246±0.024
h_T^2	0.092±0.067	0.655±0.008

Rezende et al. (2022) reported the direct heritability for the weight at 210 days 0.22 and the maternal heritability 0.08 in Limousine breed. Keeton et al. (1996) reported the direct heritability 0.25 and maternal heritability 0.19 in Limousine breed. The heritability observed in our study was higher than the value reported by Van Niekerk and Neser (2006) in Limousine breed (0.19) for weaning weight.

CONCLUSIONS

The heritability for birth weight was low and for weaning weight was high when was estimated with multivariate maternal animal model. In the selection of cattle, it is necessary the choose the best method for the genetic evaluation of the animals.

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INVESTIGATING C.260G>A MUTATION IN THE GROWTH DIFFERENTIATION FACTOR 9 GENE IN BREZNIK, BLACK-HEADED PLEVEN AND BULGARIAN DAIRY SYNTHETIC POPULATION SHEEP BREEDS

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Abstract

Growth differentiation factor 9 (GDF9) contains multiple mutations related to the fecundity in sheep. In the present experiment was studied the genetic variation in exon 1 (G1) and investigating of mutation c.260G>A, related to litter size in three sheep breeds raised in Bulgaria (Breznik, Bulgarian Dairy Synthetic Population and Black-headed Pleven). A total of 99 ewes were genotyped trough PCR-RFLP method. Results proved polymorphism in all of the three breeds. The highest genetic diversity was calculated in the BDSP population (0.385), where all three possible genotypes GG, AG and AA were identified with frequencies of 0.54, 0.41 and 0.05, respectively. The other two breeds are local breeds and as expected the genetic variation in them was lower. Genotypes GG and AG were found with very closed frequencies in both breeds. The statistical analysis manifested that all tested population were consisted with Hardy-Weinberg equilibrium.

Key words*: fecundity, GDF9, genetic diversity, polymorphism.*

INTRODUCTION

According to a report of the Food and Agriculture Organization of the United Nations (FAO), published in June 2024, world production of sheep meat is about 17.3 million which represents an increase of almost 1% compared to the amount produced in 2023. Sheep meat production takes around 5% of world meat production this year. 82% of the world's sheep meat production comes from Asian and African countries. The largest producer of sheep meat is China (5.3 million tons), followed by India (833 thousand tons), Pakistan (816 thousand tons), Turkey (762 thousand tons), Nigeria (425 thousand tons) and Algeria (363 thousand tons). Australian farmers sent more sheep and lambs to slaughterhouses in 2024 than the previous year, so Australia's sheep meat production was increased by 3.6 % (915,000 tonnes). A slight increase was expected in New Zealand (+0.7%). In 2024, a growth of 3.5% was expected in both exports and imports in the world trade in sheep meat. In 2023, Bulgaria officially produced 9361 tons of sheep and goat meat or 0.054% of the world production of lamb meat. The world consumption of lamb is increasing in parallel with the increase in population. That is why it is extremely important to find fast and cost-effective methods to develop the sheep breeding sector worldwide and more specifically to increase the number of lambs born per ewe. In 2029, the average consumption of sheep meat is expected to reach 4.2 kg per person (FAO, 2024).

The only way to ensure permanently high production of sheep meat is by maintaining high level of lambing (twins, triplets etc.). Marker assisted selection gives a fast pathway through improving process. Different QTLs have been pointed out as genetic markers associated with economically important traits. Regarding the prolificacy there are several major genes reported as eventual enhancers of litter size in sheep. One of those genes is the growth differentiation factor 9 (GDF9) (Sae-Foo et al., 2024). This gene is a member of the transforming growth factor (TGF-B) family and is located on chromosome 5. The ovine GDF9

gene spans approximately 2.5 kb and contains two exons separated by one intron. Exon 1 encodes for 134 amino acids and exon 2 for 135-456 amino acids, while the intron spans about 1126 bp (Hanrahan et al., 2004). Eight different mutations have been discovered in GDF9 (G1 to G8). They could affect the ovulation rate and even could cause infertility. Mutations G2 and G3 are located in the intron. Mutation G5 is located in exon 2 and do not result in amino acid changes. The other five mutations (G1, G4, G6, G7, and G8) lead to amino acid substitutions. Different studies announced that in G1 mutation the heterozygous genotype AG is expressed by the higher litter size per ewe (Abdelgadir et al., 2021).

The aim of the current trial is to determine the allelic variants in exon 1 (G1) c.260G>A, related to the litter size in 99 ewes from three sheep breeds raised in Bulgaria (Breznik, Bulgarian Dairy Synthetic Population and Black-headed Pleven).

MATERIALS AND METHODS

Blood sample's collection and DNA isolation

Blood samples were collected using vacuumed tubes with K_2-EDTA from 30 sheep of Breznik (Br) sheep breed, 30 sheep of Black-headed Pleven (BHP) and 39 sheep of Bulgarian Dairy Synthetic Population from 3 different distinct flocks raised in Bulgaria. DNA was obtained from the whole blood taken from *vena jugularis* using a DNA purification kit QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

The blood collection was in accordance with the requirements for the humane treatment of animals listed in the regulatory documents of the Republic of Bulgaria and the European Union. All DNA analysis procedures were performed in the Laboratory of Genetics of the Faculty of Agronomy at the University of Forestry, Sofia.

DNA analysis

The extracted DNA was further processed by PCR analysis. A 462 bp fragment of the GDF9 gene exon 1 was amplified by polymerase chain reaction (PCR) in all tested samples using the following primers (Hanrahan et al., 2004)
Forward:
5′GAAGACTGGTATGGGGAAATG3′ and
Reverse:
5′CCAATCTGCTCCTACACACCT3′.

PCR reactions were performed in a final volume of 25 µl, including 12.5 µl Red Taq DNA polymerase master mix (2x) (VWR), 1 µl of each primer (10 pmol/µl) (Bioneer), 10 µl DNA template and distilled water to final volume. PCR conditions were performed as described: primary denaturation at 94°C for 5 minutes, followed by 30 cycles a denaturation at 94°C for 1 minute, annealing at 63°C for 45 s, extension at 72°C for 1 minutes, and final extension at 72°C for 10 minutes. The PCR procedure was performed in a VerityPro 96-Well Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific).

Genotyping of the GDF9 gene

All PCR products were subjected to restriction with *HhaI* enzyme. The reactions were incubated at 37°C for 20 minutes and then inactivated at 65°C for 10 minutes. The restriction mixture was prepared in a final volume of 20 µL, containing 1 µL (5 U) of restriction enzyme *HhaI* (cat. no.ER1851, Thermo Fisher Scientific), 5 µL ddH_2O and 2 µL Tango Buffer). After digestion, samples were run in 2.5% agarose gel electrophoresis for 60 min in 120 V and 80 mA. The genotypes were determined under UV transilluminator (Hi-UVTM Duo Capture, HIMEDIA).

Statistical analysis

Statistical software was used to determine genotype and allele frequencies, observed and expected heterozygosity, coefficient of inbreeding for the studied locus (Yeh et al., 1997). The chi-square test was used to analyze whether the studied population were in Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSIONS

A total of 99 ewes were genotyped trough PCR-RFLP method. Results proved polymorphism in all of the three breeds (Table 1). In all samples were determined the PCR products with expected length of 462 bp (Figure 1). The highest genetic diversity was calculated in the Bulgarian Dairy Synthetic Population (0.385), where all three possible genotypes GG, AG and AA were identified with frequencies of 0.54,

0.41 and 0.05, respectively (Figure 2). In ewes from Breznik and Black-headed Pleven sheep breeds the genetic variation was lower as expected. This could be explained with the fact that those two breeds are local Bulgarian breeds and they are more or less stable and conservative. Genotypes GG and AG were found with very closed frequencies in both breeds. Homozygous genotype AA was not observed in those two local Bulgarian sheep breeds.

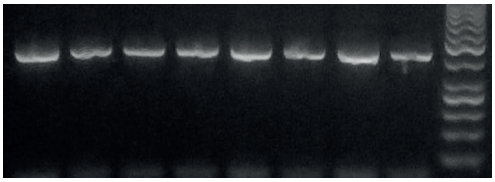


Figure 1. Electropherogram of PCR products of GDF9 with length of 462 bp

In Table 1 was clearly demonstrated that the genetic diversity (Ho) was higher in the herd of Bulgarian Dairy Synthetic population sheep breed (0.410) The statistical analysis manifested that all tested population were consisted in Hardy-Weinberg equilibrium. The coefficient of inbreeding (Fis) is the proportion of the variance in the population contained in an individual. A high Fis implies a considerable degree of inbreeding. In the present study all breeds had a negative degree of Fis which meant that despite the lower genetic diversity for the tested locus in the two local breeds the implemented selection aimed to avoid inbreeding.

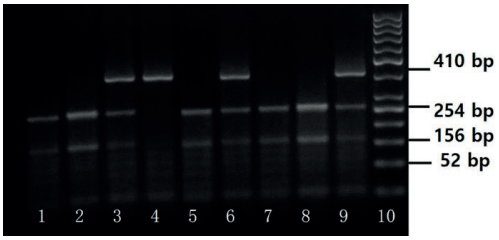


Figure 2. Separated fragments and different genotypes pattern in ewes from Bulgarian Diary Synthetic Population

On Figure 2 were showed the results from fragment separation in Bulgarian Dairy Synthetic Population where all three genotypes could be seen. The homozygous genotype GG was represented by 3 bands with lengths of 254 bp, 156 bp and 52 bg. The heterozygous genotype AG revealed 4 fragments with lengths 410 bp, 245 bp, 156 bp and 52 bp. The homozygous genotype AA demonstrated 2 bands with lengths of 410 bp and 52 bp.

Sheep (*Ovis aries*) are the most widely farmed animal species in many parts of the world. There are over 1300 sheep breeds worldwide. Sheep are multipurpose animals that are raised for their meat, milk, fleece, skin, fur and fertilizer (Koyun et al., 2021).

The crucial factor in sheep breeding is the litter sized. The reproduction potential is determined by breed, environment and most of all by genetics (Hassooni and Zamit, 2024).

The growth differentiation factor 9 is one of the most studied genes worldwide associated with the prolificacy in sheep. PCR-RFLP technique appears to be easy and fast method for genotyping and identification of allelic variants of this gene. Kırıkçı (2022) studied 50 Akkaraman sheep in order to establish the genetic variability in G1 of GDF9 gene. The author reported very similar result to these in Black-headed Pleven and Breznik sheep breeds in the present study. In Akkaraman animals were determined genotypes GG and AG with frequencies 0.87 and 0.13 respectively, and genotype AA was not found.

A study on indigenous sheep in Bangladesh Results suggested that the genotypes had a significant effect on litter size. The study also showed that the homozygous genotype GG had the lowest litter size (1.59±0.09) and the homozygous genotype AA had the highest litter size (2.00±0.41) (Hossain et al., 2020), which was in agreement to a previous our work regarding G1 mutation in GDF9 in ewes from Bulgarian Dairy Synthetic population breed where a proven higher fertility was observed in ewes born as twins with genotype AA (1.97) compared to twins born as carriers of heterozygous genotype AG (1.65) ($p \leq 0.05$) (Dimitrova et al., 2024).

Conversely - in a study by Kasiriyan et al. (2011) in 150 Sangsari sheep, it was found that sheep with a homozygous mutant genotype AA were fertile, but had the smallest litter size compared to those homozygous for the wild allele GG and heterozygous AG ewes.

Our research team has conducted a massive study on different sheep breeds raised in

Bulgaria regarding productive traits in sheep including fecundity. In a previous study of the GDF9 gene in Bulgarian indigenous sheep breeds, a genetic variation was detected. Homozygous genotype GG and heterozygous genotype AG were identified in the Cooper-Red Shumen sheep breed with frequencies 0.97 and 0.03, respectively. In the Karakachan sheep breed were determined all three genotypes GG, AG, and GG with frequency 0.76, 0.17, and 0.07, respectively (Bozhilova-Sakova and Dimitrova, 2021).

Other investigation of some Merino sheep breeds in Bulgaria, showed also that GDF9 locus was polymorphic. In Ascanian and Caucasian Merino, were detected genotypes GG and AG with similar frequencies of 0.90 and 0.10, respectively. In the Karnobat Merino breed were revealed the three genotypes GG, AG, and AA with frequencies 0.70, 0.27, and 0.03, respectively (Dimitrova et al., 2020). In 60 ewes from Northeast Bulgarian Merino sheep breed the GDF9 gene was identified as polymorphic with presence of two alleles G and A with frequencies 0.94 and 0.06 and genotypes GG and AG with frequencies 0.88 and 0.12, respectively (Bozhilova-Sakova and Dimitrova, 2020).

In disagreement to the present investigation Al-Mutar and Younis (2020) tested the effect of point mutation in the Growth Differentiation Factor 9 Gene of oocytes on the sterility and fertility of Awassi sheep. They announced that the mutant allele A in G(199)A locus was demonstrated in sterile samples, while the wild G allele was detected in fertile ewes. The ovarian tissues taken from the non-fertile carriers of the mutant allele A showed severe hypoplastic changes. Hypoplasia was characterized by diminished developmental follicles mostly replaced with stromal connective tissue.

In an experiment conducted by Aboelhassan et al. (2021), the authors studied the different variants of GDF9 in Egyptian sheep, using the T-ARMS-PCR technique to calculate the effect of genetic variability on reproductive traits, the average litter size, and twining in particular. According to the results, the GDF9 was identified as the most important genetic marker for improving fertility in Egyptian sheep.

All stated so far prove that growth differentiation factor 9 gene was polymorphic in the investigated sheep breeds worldwide and hence it is suitable for conducting in marker assisted selection aimed to improving reproduction in sheep.

Table 1. Effective allele number, allele and genotype frequencies, heterozygosity, coefficient of inbreeding, chi-square and p-value of the tested breeds

Breed	n	Ae	Allele frequencies		Genotype frequencies			Heterozygosity		Fis	X2	p
			G	A	GG	AG	AA	Ho	He			
BDSP	39	1.63	0.74	0.26	0.54	0.41	0.05	0.410	0.385	-0.064	0.4	NS*
BR	30	1.10	0.95	0.05	0.90	0.10	0.00	0.100	0.095	-0.053	0.0	NS*
BHP	30	1.15	0.93	0.07	0.87	0.13	0.00	0.133	0.130	-0.023	0.0	NS*

*statistically non-significant difference

CONCLUSIONS

The presence of polymorphism was found in all three studied breeds. The highest genetic diversity was observed in sheep from Bulgarian Dairy Synthetic Population, where all three possible genotypes were found.

The two local breeds (Breznik and Black-headed Pleven) are characterized by lower genetic diversity and the presence of two genotypes (GG and AG).

The results found are probably due to the different breeding systems, as the local breeds are bred extensively, with continuous application of "maintaining" selection in order to preserve the typical characteristics of the breed, and on the other hand the number of animals studied is limited.

Additional studies are needed, including larger sample sizes, to confirm the current results.

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RESEARCHES REGARDING THE OPRIMIZATION OF THE MILK RECORDING IN ROMANIAN SPOTTED CATTLE BREED

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Abstract

This study aimed to determine the optimal number of records required to estimate the breeding value for milk production in Romanian Spotted cattle during the first and second lactations. Data from 9,235 test-day records (1,270 cows) for the first lactation and 9,984 records (1,409 cows) for the second were analyzed using the Best Linear Unbiased Prediction (BLUP) method in a Test-Day Random Regression model. Heritability estimates for milk yield, fat, and protein content ranged from 0.194 to 0.381 in the first lactation and 0.184 to 0.372 in the second. Although 10 test-day records were initially considered, results indicated that focusing on the first five intervals was sufficient, as they showed higher heritability. Reducing the number of test-day records to those with the highest genetic determination improves selection accuracy while lowering the costs of official milk performance recording. This streamlined approach enhances breeding efficiency, supporting genetic progress in Romanian Spotted Simmental cattle.

Key words*: BLUP, heritability, milk recording, regression model, Test-Day.*

INTRODUCTION

The genetic evaluation and selection of dairy cattle heavily rely on milk recording, which provides crucial information for calculating breeding values and enhancing overall production efficiency. For the Romanian Spotted breed, it is essential to optimize the number of milk recording tests to improve selection accuracy while minimizing performance recording expenses. An excessive number of test-day records may lead to increased costs without significantly improving genetic evaluations, whereas too few records could reduce the accuracy of selection (Pelmuș et al., 2021; Davidescu et al., 2023). Studies have shown that selecting the most informative test-day (Jensen, 2001) intervals can enhance the efficiency of genetic evaluations while maintaining reliable heritability estimates (Cecchinato et al., 2015). Furthermore, optimizing milk recording frequency can contribute to more sustainable breeding programs by reducing labor and testing costs while preserving genetic progress (Interbull, 2014).

The Romanian Spotted cattle, also referred to as Romanian Spotted Simmental, is a versatile breed raised for both dairy and beef production. This breed has adapted well to Transylvania's climate, where it is mainly found, as well as in Banat and North-Eastern Moldova. Its ability to thrive in these regions makes it a valuable asset to Romanian agriculture. Beyond its adaptability, the breed is known for its good milk yield, balanced fat and protein content, and favorable feed efficiency (Gantner et al., 2009). Moreover, the dual-purpose nature of Romanian Spotted cattle ensures its economic sustainability, as it contributes significantly to both milk and meat production sectors (Nistor et al., 2011). Ongoing genetic improvement programs aim to enhance production traits while maintaining the breed's resilience to environmental conditions and disease resistance.

Due to its strong genetic heritage, the breed exhibits high resilience to local environmental factors, including temperature variations and diverse feeding conditions. Romanian Spotted cattle are known for their adaptability to extensive and semi-intensive farming systems, making them a valuable asset to local farmers who seek sustainable and efficient livestock management. Their ability to maintain stable production levels under different management

conditions highlights their economic importance (Rotar et al., 2020). Additionally, the breed demonstrates strong resistance to common diseases affecting dairy and beef cattle, reducing the need for veterinary interventions and contributing to lower production costs (Strabel & Misztal, 1999). These traits make Romanian Spotted cattle an ideal choice for small- and medium-scale farms, ensuring long-term productivity while aligning with sustainable agricultural practices.

The enhancement of dairy cows through genetic selection for characteristics such as milk yield, milk fat content, and breeding efficiency is influenced by various factors within the herd under evaluation. The genetic improvement process is influenced by several key elements. These include the range of observable and genetic diversity, the degree to which these characteristics are inherited, and the relationships between them. Moreover, the total productive capability of the evaluated cattle group is a significant factor in this genetic enhancement endeavor. In addition to genetic parameters, environmental factors such as nutrition, herd management, and climate conditions also play a crucial role in shaping production traits (García-Ruiz et al., 2016). Advances in genomic selection and marker-assisted breeding have further improved the accuracy of selecting animals with superior traits, accelerating genetic progress (Jamrozik et al., 1998). Furthermore, maintaining a balance between production efficiency and animal welfare is essential to ensure sustainable genetic improvement programs that benefit both farmers and livestock (Kaygisiz, 2013).

The use of test-day random regression models has been widely adopted in developed countries for genetic evaluation due to its ability to increase selection accuracy by utilizing multiple records and reduce production control costs while allowing for earlier selection (Jakobsen et al., 2002; Ptak & Schaeffer, 1993). Previous research has demonstrated that genetic parameters, such as heritability, fluctuate across different stages of lactation. Understanding these variations allows for the identification of the most informative test-day intervals, which can improve the precision of selection while minimizing unnecessary data collection. Moreover, random regression models provide a more detailed analysis of individual lactation curves, capturing genetic variability more effectively than traditional models (Strabel and Misztal, 1999). The application of the Best Linear Unbiased Prediction (BLUP) methodology in combination with Test-Day Random Regression Models has been widely used to analyze such phenotypic data, providing valuable insights into the genetic potential of dairy cattle (Grosu et al., 2019). These advancements contribute to more efficient breeding programs, ultimately enhancing the productivity and sustainability of dairy farming (Gantner et al., 2009).

This study aims to assess the optimal number of test-day records required for the estimation of breeding values in Romanian Spotted cattle during the first and second lactations. By analyzing a comprehensive dataset using advanced statistical models, we seek to determine the most effective recording strategy that maximizes genetic gain while reducing operational costs. Identifying the most informative test-day intervals is crucial for improving selection accuracy while minimizing redundant data collection. Additionally, optimizing milk recording frequency can enhance the cost-effectiveness of genetic evaluation programs and ensure a more sustainable approach to dairy cattle breeding (Pandey & Rajak, 2020). The findings of this research are expected to contribute to the refinement of milk recording protocols, ultimately benefiting dairy farmers and breeding programs through improved efficiency and selection accuracy. Moreover, integrating refined test-day models with genomic selection approaches could further accelerate genetic progress in dual-purpose breeds such as the Romanian Spotted cattle (Buaban et al., 2020).

MATERIALS AND METHODS

Two lactations were taken into account. From first lactation was analyzed a sample of 9,235 test recording belonging to 1,270 Romanian Spotted Simmental cows, in first lactation. From second lactation a sample of 9,984 test recording made of 1,409 of the same breed.

To determine the optimal number of controls for calculating the breeding value over a 5-305 day lactation period, we considered 10 controls.

The statistical analysis of the data was conducted using R software (version 4), employing the BLUP methodology within the Test Day Random Regression Model.
This study aimed to determine the optimal number of controls required for calculating the breeding value in the Romanian Spotted Cattle population.
The following biometrical model was used to describe the performances at the monthly control level:

$$y_{itklm} = HYS_l + AGE_m + DIM_t + \sum_{k=0}^{q}(\beta \cdot z_{itk}) + \sum_{k=0}^{q}(\alpha_{ik} \cdot z_{itk}) + \sum_{k=0}^{q}(\gamma_{ik} \cdot z_{itk}) + e_{itklm} \quad [1]$$

where:
y_{itklm} is the observation of cow i measured at time t within herd-year-season (HYS), subclass l and age (AGE) subclass m;
DIM_t is the fixed effect of DIM clases;
b is the fixed covariate effect on the ith cow;
α_{ik} and γ_{ik} are the kth random regression of additive genetic and permanent environmental effects for cow i, respectively;
z_{itk} is the kth order of LPs/P-Spline/NCS for cow i measured at DIM_t, DIM_t denote the order of LPs;
eitklm is the random residual.
The covariates are customized, case by case, depending on the biometric model used and the function used (Legendre polynomial, P-Spline or Natural cubic Spline functions).
In matrix notation, the model [1] can be rewritten as follows:

$$y = X_1b_1 + X_2b_2 + X_3b_3 + X_4\beta + Z_\alpha + Z_\gamma + e \quad [2]$$

where:
y = vector of TD milk yield record;
X_1= incidence matrix of fixed effect for HYS;
b_1 = vector of fixed effect for HYS;
X_2= incidence matrix of fixed effect for Age of calving;
b_2 = vector of fixed effect for Age of calving;
X_3= incidence matrix of fixed effect for Days in Milk;
b_3 = vector of fixed effect for Days in Milk (DIM);
X_4 = covariate matrix for Days in Milk (DIM);
β = fixed regression coefficients;
Z_1= covariates matrix for all animals;
α = random regression coefficients for the breeding value;
Z_2 = covariates matrix for cows with records;
γ = random regression coefficients for the permanent environmental effect;
e = vector of residual effects.
The system of equations corresponding to model [2] is as follows:

$$\begin{bmatrix} X^T \cdot X & X^T \cdot Z_1 & X^T \cdot Z_2 \\ {Z_1}^T \cdot X & {Z_1}^T \cdot Z_1 + \sigma_e^2 \cdot (A^{-1} \otimes G^{-1}) & {Z_1}^T \cdot Z_2 \\ {Z_2}^T \cdot X & {Z_2}^T \cdot Z_1 & {Z_2}^T \cdot Z_2 + \sigma_e^2 \cdot (I \otimes P^{-1}) \end{bmatrix} \cdot \begin{bmatrix} \tilde{b}_1 \\ \hat{\alpha} \\ \gamma \end{bmatrix} = \begin{bmatrix} X^T \cdot y \\ Z_1 \cdot y \\ {Z_2}^T \cdot X \end{bmatrix}$$

Where: X= (X_1, X_2, X_3, X_4).

The (co)variance structure was assumed for random effects of model:

$$V = \begin{bmatrix} A \otimes G & 0 & 0 \\ 0 & I \otimes P & 0 \\ 0 & 0 & I\sigma_e^2 \end{bmatrix}$$

where:
Var (a) =$A \otimes G$;
Var (a) = additive variance;
Where $\otimes$ is Kronecker product function;
Var (p) = $I \otimes P$;
Var (p) = environmental variance;
G and P are the matrices of genetic and permanent environmental variances and covariances between random regression coefficients.
I = represents the identity matrix with the size equal with the number of cows with records;
σ_e^2= residual variance for lactation assumed to be constant throughout the lactation.

RESULTS AND DISCUSSIONS

A study on milk production in Romanian Spotted cattle during their first lactation showed an average daily milk yield of 18.76 kg, with fat and protein yields of 0.77 kg and 0.65 kg, respectively (Table 1).

Table 1. Average milk production performance of the Romanian Spotted Cattle breed for the first lactation

COP	n	Milk (kg) $\overline{X} \pm S_{\overline{X}}$	Fat (kg) $\overline{X} \pm S_{\overline{X}}$	Protein (kg) $\overline{X} \pm S_{\overline{X}}$
1	862	21.12±0.198	0.87±0.010	071±0.007
2	908	21.18±0.206	0.85±0.010	0.70±0.008
3	914	20.81±0.202	0.84±0.009	0.69±0.007
4	947	19.91±0.199	0.81±0.009	0.67±0.007
5	997	18.93±0.189	0.77±0.009	0.65±0.007
6	1,003	18.38±0.186	0.75±0.008	0.64±0.007
7	976	17.70±0.189	0.73±0.008	0.62±0.007
8	980	17.02±0.181	0.71±0.008	0.61±0.007
9	900	16.37±0.196	0.68±0.009	0.59±0.007
10	748	15.97±0.214	0.67±0.009	0.58±0.008
Average		18.76±0.195	0.77±0.009	0.65±0.007

The research utilized 9,235 test-day records, which demonstrated the breed's milk production capabilities. The results align with previous research conducted on comparable cattle groups. For instance, a study by Pelmuș et al. (2021) observed that the Romanian Spotted breed yielded an average milk production of 5,545 kg during a 305-day lactation cycle.

For the second lactation, the study evaluated 9,984 test-day records, where the mean daily milk yield increased to 19.76 kg, with fat and protein yields reaching 0.8 kg and 0.68 kg, respectively.

These values suggest a natural increase in milk production with lactation progression, a common trend observed in dairy cattle.

Table 2 has shown the average milk production performances of the breed, for the second lactation Romanian Spotted Cattle Breed.

Table 2. Average milk production performance of the Romanian Spotted Cattle breed for the second lactation

COP	n	Milk (kg) $\overline{X} \pm S_{\overline{X}}$	Fat (kg) $\overline{X} \pm S_{\overline{X}}$	Protein (kg) $\overline{X} \pm S_{\overline{X}}$
1	910	24.14±0.237	0.97±0.011	0.82±0.009
2	1,014	23.25±0.224	0.92±0.010	0.77±0.008
3	1,036	22.27±0.228	0.89±0.010	0.75±0.008
4	1,061	21.18±0.22	0.84±0.010	0.72±0.008
5	1,070	20.00±0.205	0.80±0.009	0.69±0.007
6	1,035	19.29±0.212	0.78±0.009	0.67±0.008
7	1,055	18.02±0.195	0.73±0.008	0.64±0.007
8	1,053	17.17±0.189	0.71±0.008	0.61±0.007
9	958	16.25±0.192	0.68±0.008	0.59±0.007
10	792	15.38±0.204	0.64±0.009	0.56±0.008
Average		19.76±0.210	0.8±0.009	0.68±0.008

When comparing these values with results reported for other Simmental breeds, Croatian Simmental cows (Gantner et al., 2009) exhibited similar performance, while Italian Simmental cattle (Cecchinato et al., 2015; Ablondi et al., 2023) had higher daily milk yields, ranging between 22.56 kg and 25.4 kg. This discrepancy may be attributed to differences in genetic selection, feeding management, and environmental conditions across different countries.

Comparable findings were reported by Gantner et al. (2009), who observed fat (0.75 kg) and protein (0.63 kg) yields akin to those in the current research. The genetic relationships among milk yield, fat yield, and protein yield were evaluated based on earlier investigations. In Holstein cattle, Hammami et al. (2008) discovered strong genetic correlations (0.98-0.99) between milk and protein yields, with slightly lower correlations (0.79-0.96) between fat and protein yields.

These results indicate that selection for one trait is likely to affect the others. Likewise, Buaban et al. (2020) noted moderate genetic correlations in Thai dairy cattle, with values ranging from 0.56 to 0.69 for milk-fat and milk-protein relationships. The calculation of genetic, environmental, and phenotypic variance components offered important insights into the heritability of milk production characteristics. The values of the causal components of phenotypic variance and heritability are presented in Table 3 for first lactation.

Table 3. The causal components of phenotypic variance and heritability for first lactation

Milk record nr.	Genotypic variance	Permanent environmental variance	Error variance	Phenotypic variance	Heritability
1	10.32	4.13	18.41	32.86	0.314
2	7.4	2.96	16.9	27.27	0.272
3	7.23	2.89	20.53	30.66	0.236
4	7.8	3.12	19.81	30.73	0.254
5	5.5	2.2	20.69	28.39	0.194
6	7.74	3.1	19.97	30.8	0.251
7	6.92	2.77	19.16	28.85	0.24
8	7.09	2.84	19.92	29.84	0.238
9	9.98	3.99	18.83	32.8	0.304
10	16.2	6.48	19.82	42.5	0.381

Genotypic variance ranged from 5.5 to 16.2, reflecting substantial genetic differences among individuals.

Permanent environmental variance was between 2.2 and 6.48, indicating the influence of environmental factors across multiple lactations.

Error variance was estimated between 16.9 and 20.69, representing unexplained variability.

Phenotypic variance varied from 27.27 to 42.5, capturing the combined effects of genetic and environmental contributions.

Heritability estimates ranged from 0.194 to 0.381, classifying these traits as moderately heritable.

The findings indicate that milk yield is genetically influenced but also significantly affected by environmental conditions. Higher heritability in the first five test-day intervals suggests that fewer but strategically chosen controls may be sufficient for accurate selection decisions.

A comparable trend was observed during the second lactation, where:

Genotypic variance ranged from 8.71 to 25.68, exhibiting a slight decrease compared to the first lactation.

Permanent environmental variance fluctuated between 3.48 and 10.27, demonstrating relative stability.

Error variance remained within the range of 30.01 to 35.69.

Phenotypic variance was recorded between 46.44 and 69.04, aligning with values observed in the first lactation.

Heritability estimates were slightly lower, ranging from 0.184 to 0.372, while still indicating a moderate genetic influence.

The values of the causal components of genetic, phenotypic, environmental variance and heritability are presented in Table 4 for the second lactation.

Table 4. The causal components of phenotypic variance and heritability for second lactation

Milk record no.	Genotypic variance	Permanent environmental variance	Error variance	Phenotypic variance	Heritability
1	16.36	6.54	32.14	55.04	0.297
2	11.74	4.69	30.01	46.44	0.253
3	11.47	4.59	35.69	51.74	0.222
4	12.36	4.95	34.94	52.25	0.237
5	8.71	3.48	35.09	47.28	0.184
6	12.27	4.91	33.74	50.91	0.241
7	10.97	4.39	33.3	48.67	0.225
8	11.24	4.5	33.57	49.3	0.228
9	15.81	6.32	32.92	55.05	0.287
10	25.68	10.27	33.09	69.04	0.372

Similar to the findings from the first lactation, the results suggest that prioritizing early test-day intervals represents an effective approach for optimizing recording strategies, reducing costs, and preserving selection accuracy.

Optimization of Test-Day Controls

The graphical analysis of genetic variance across test-day intervals revealed a distinct trend, indicating that heritability was highest during the first five test-day intervals. This finding suggests that genetic progress is directly influenced by the heritability of the analyzed trait. Specifically, the observed heritability values ranged from 0.194 to 0.381, implying that among the 10-11 recorded test-day intervals, focusing on the first 1-5 intervals would be sufficient due to their higher heritability (Figure 1). A similar pattern was observed in the second lactation, where heritability values ranged from 0.184 to 0.372, reinforcing the conclusion that the early test-day intervals provide the most genetically informative data (Figure 2). This trend is further illustrated in the graphical representation below, supporting the notion that optimizing the number of test-day records can enhance selection efficiency while minimizing recording efforts (Figures 1 and 2).

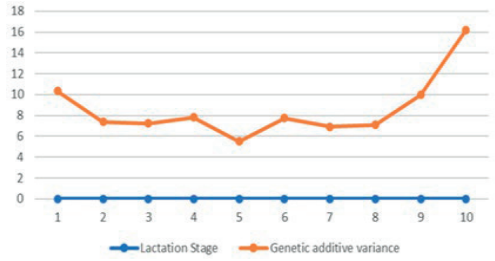


Figure 1. The evolution of additive genetic variance between 5-305 days in milk (DIM)

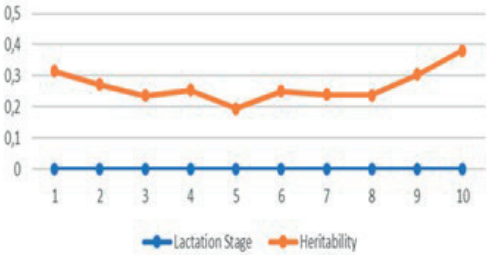


Figure 2. The evolution of heritability between 5-305 days in milk (DIM)

Recording milk yield during the first five test-day intervals provides the most genetically informative data for selection. Limiting test-day recordings beyond this threshold can lead to a substantial reduction in costs while maintaining selection accuracy.

CONCLUSIONS

This study aimed to determine the optimal number of test-day milk recordings required for accurate breeding value estimation in Romanian Spotted cattle during the first two lactations. The findings indicate that heritability estimates ranged from 0.194 to 0.381 in the first lactation and from 0.184 to 0.372 in the second, confirming a moderate genetic influence on milk yield, fat, and protein content. Notably, the analysis demonstrated that the first five test-day records provide sufficient genetic information for selection, as these intervals exhibit the highest heritability values. This strategic approach ensures accurate genetic evaluations while significantly reducing the costs associated with official performance recording.

Furthermore, the consistent pattern of higher heritability in early lactation suggests that genetic progress in milk production traits can be effectively achieved by focusing on the most informative test-day intervals. By refining traditional milk recording methods and integrating statistical models such as Best Linear Unbiased Prediction (BLUP), this research offers valuable insights for improving selection efficiency in the Romanian Spotted breed. These findings may also serve as a reference for optimizing milk recording systems in other dairy cattle breeds. Future research should further explore the impact of reduced testing frequencies on long-term genetic gains and the economic sustainability of dairy farming.

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RESEARCHES ON MODELING OF THE LACTATION CURVE IN ROMANIAN SPOTTED SIMMENTAL CATTLE BREED

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Abstract

Milk production in dairy cattle is influenced by multiple genetic and environmental factors, making precise lactation curve modeling essential for accurate milk yield estimation and genetic evaluation. In recent decades, numerous scientific studies have focused on improving these models to enhance the accuracy of milk production estimates for standard lactation. This study aims to identify the most suitable model for predicting breeding values using a limited number of test-day records at different lactation stages. Data from two lactations were analyzed. For the first lactation, 9235 test records from 1270 Romanian Spotted Simmental cows were examined, while the second lactation included 9984 test records from 1409 cows of the same breed. Production and pedigree data were modeled using three biometric functions: Legendre Orthogonal Polynomial, Natural Cubic Spline, and P-Spline Function. Among these, the P-Spline Function provided the most accurate results. These findings are particularly valuable in animal breeding, enabling more precise genetic evaluations and contributing to improved selection strategies for dairy cattle.

Key words*: genetic progress, heritability, optimization of genetic selection, P-Spline, Test-day model.*

INTRODUCTION

The prediction of breeding values in cattle based on milk yield recorded during monthly official production control is significantly more accurate than systems relying on total milk yield over a standard lactation period of 305 days. This increased accuracy is due to the fact that monthly test-day records (every 28 days, method A4) allow for performance corrections based on environmental factors acting at the time of phenotype expression. In contrast, when using the total 305-day milk yield, it is assumed that the same environmental conditions persist throughout lactation, which may lead to biased genetic evaluations (White et al., 1999; Druet et al., 2003). Furthermore, test-day models provide a more dynamic representation of lactation performance, capturing short-term variations caused by factors such as feeding strategies, health status, and seasonal influences (Misztal, 2006). By incorporating these variations, test-day models enhance the accuracy of genetic evaluations, reducing the risk of overestimating or underestimating an animal's true genetic potential (Macciotta et al., 2005; Bohmanova et al., 2008). Consequently, they have become a standard approach in modern dairy cattle breeding programs, facilitating more precise selection decisions and accelerating genetic progress (Silvestre et al., 2010; Grosu & Rotar, 2015).

The mathematical model that incorporates monthly test-day records is referred to as the "test-day" model. Such models require mathematical functions capable of accurately describing the evolution of the lactation curve from calving to the end of lactation. These models are important in accurately capturing the dynamics of milk production, as they reflect the varying environmental conditions and physiological changes that occur throughout lactation. Over time, various biometric models have been developed, ranging from nonlinear parametric functions (Wilmink, 1987; Ali & Schaeffer, 1987) to orthogonal Legendre polynomials, spline-based functions, and fractional polynomials (Macciotta et al., 2005; 2008; 2010; Silvestre et al., 2005; Silvestre et al., 2010; Misztal, 2006; Bohmanova et al., 2008). These models enable more precise

estimation of breeding values, as they allow for the incorporation of genetic and environmental factors in a more accurate manner than traditional models based on total lactation yield. Furthermore, the flexibility of these models to accommodate different lactation patterns has made them a valuable tool in genetic evaluations and selection decisions in dairy cattle breeding (Jensen, 2001; Lidauer et al., 2003; Macciotta et al., 2011; McCarthy & Veerkaamp, 2012; Swalve, 1995; Wahinya et al., 2020).

In a previous study, the authors found that among the ten monthly test-day records, the highest heritability estimates were observed between the 4th and 8th test-day records. Since genetic progress is directly proportional to heritability, these findings suggest that the number of test-day records required for accurate genetic evaluation could be reduced from ten to five, focusing on the middle of the lactation period (Silvestre et al., 2010; Macciotta et al., 2008). This result is consistent with other studies showing that heritability tends to be higher during the peak of lactation, which is typically reached around the 4th to 8th test-day. Furthermore, by reducing the number of test-day records, the efficiency of genetic evaluation is enhanced, leading to more cost-effective breeding programs without sacrificing accuracy (Druet et al., 2003; Bohmanova et al., 2008). Thus, optimizing the number of test-day records is essential for improving genetic selection in dairy cattle breeding programs.

Given this, the next challenge is identifying the optimal mathematical function that can accurately predict breeding values from a reduced number of test-day records. Selecting the most suitable function is important as it ensures precise genetic evaluations with minimal data. Several mathematical functions have been proposed in the literature, each with its strengths in fitting the lactation curve. In this study, three mathematical functions were investigated: Legendre Orthogonal Polynomials, P-Spline Function, and Natural Cubic Spline Function. These models have been widely used due to their ability to represent the complex, nonlinear nature of lactation curves and to accommodate irregular test-day records (Macciotta et al., 2005; Misztal, 2006; Bohmanova et al., 2008). Among these, the P-Spline function has been reported to provide the best balance between model complexity and prediction accuracy (White et al., 1999; Bohmanova et al., 2008). Therefore, understanding the advantages of each function is essential for improving genetic evaluations and optimizing selection strategies in dairy cattle breeding.

MATERIALS AND METHODS

Two lactations were analyzed in this study. The first lactation included a sample of 9,235 test-day records from 1,270 Romanian Spotted Simmental cows. For the second lactation, a sample of 9,984 test-day records was taken from 1,409 cows of the same breed. The trait analyzed was the quantity of milk, measured and recorded during the first 10 months of lactation for both lactations 1 and 2.

To achieve the study's objective, seven working variants were considered. One control variant was used, which involved 10 test-day records per cow, while six experimental variants were examined with different sets of test-day controls: from 1 to 5; from 2 to 6; from 3 to 7; from 4 to 8; from 5 to 9; and from 6 to 10.

To model the evolution of the lactation curve, three mathematical functions were employed: the Legendre Orthogonal Polynomial, the P-Spline function, and the natural cubic Spline function. Calculations were performed using an R script written by H. Grosu.

The following biometric model was used to describe the performance at the monthly control level:

$$y_{itklm} = HYS_l + AGE_m + DIM_t + \sum_{k=0}^{q}(\beta \cdot z_{itk}) + \sum_{k=0}^{q}(\alpha_{ik} \cdot z_{itk}) + \sum_{k=0}^{q}(\gamma_{ik} \cdot z_{itk}) + e_{itklm} \quad [1]$$

where:

y_{itklm} is the observation of cow i measured at time t within herd-year-season (HYS), subclass l and age (AGE) subclass m;

DIM_t is the fixed effect of DIM clases;

b is the fixed covariate effect on the ith cow;

α_{ik} and γ_{ik} are the *kth* random regression of additive genetic and permanent environmental effects for cow *i*, respectively;

z_{itk} is the *kth* order of LPs/P-Spline/NCS for cow i measured at DIM_t, DIM_t denote the order of LPs;

e_{itklm} is the random residual.

The covariates are customized, case by case, depending on the biometric model used and the function used (Legendre polynomial, P-Spline or Natural Cubic Spline functions).
In matrix notation, the model [1] can be rewritten as follows:

$$y = X_1b_1 + X_2b_2 + X_3b_3 + X_4\beta + Z_\alpha + Z_\gamma + e \quad [2]$$

where:
y = vector of test-day (TD) milk yield records;
X_1= incidence matrix for the fixed effect of herd-year-season (HYS);
b_1 = vector of fixed effect for HYS;
X_2= incidence matrix of fixed effect for age at calving;
b_2 = vector of fixed effect for age at calving;
X_3= incidence matrix of fixed effect for days in milk (DIM);
b_3 = vector of fixed effect for days in milk;
X_4 = covariate matrix for days in milk;
β = vector of fixed regression coefficients;
Z_1= covariates matrix for all animals;
α = vector of random regression coefficients for the breeding value;
Z_2 = covariates matrix for cows with records;
γ = vector of random regression coefficients for the permanent environmental effect;
e = vector of residual effects.
The system of equations corresponding to model [2] is as follows:

$$\begin{bmatrix} X^T \cdot X & X^T \cdot Z_1 & X^T \cdot Z_2 \\ {Z_1}^T \cdot X & {Z_1}^T \cdot Z_1 + \sigma_e^2 \cdot (A^{-1} \otimes G^{-1}) & {Z_1}^T \cdot Z_2 \\ {Z_2}^T \cdot X & {Z_2}^T \cdot Z_1 & {Z_2}^T \cdot Z_2 + \sigma_e^2 \cdot (I \otimes P^{-1}) \end{bmatrix} \cdot \begin{bmatrix} \tilde{b}_1 \\ \hat{\alpha} \\ \gamma \end{bmatrix} = \begin{bmatrix} X^T \cdot y \\ Z_1 \cdot y \\ {Z_2}^T \cdot X \end{bmatrix}$$

Where: X = (X_1, X_2, X_3, X_4).

The (co)variance structure was assumed for random effects of model:

$$V = \begin{bmatrix} A \otimes G & 0 & 0 \\ 0 & I \otimes P & 0 \\ 0 & 0 & I\sigma_e^2 \end{bmatrix}$$

where:
Var (a) = A ⊗ G;
Var (a) = additive variance;
Where ⊗ is Kronecker product function;
Var (p) = I ⊗ P;
Var (p) = environmental variance;
G and P are the matrices of genetic and permanent environmental variances and covariances between random regression coefficients.
I = represents the identity matrix with the size equal with the number of cows with records;
σ_e^2= residual variance for lactation assumed to be constant throughout the lactation.
The three models were compared according to the residual variances and also to the correlation between the breeding values (Grosu & Rotar, 2015).

RESULTS AND DISCUSSIONS

Average population performances

In Table 1 summarizes the key data on milk production performance and heritability estimates for the Romanian Spotted breed during their first two lactations. This data provides valuable insights into the genetic potential of the breed, highlighting both the average milk yield and the heritability values observed across the two lactation periods. Understanding these factors is important for optimizing breeding programs, as heritability estimates reflect the proportion of variation in milk production that can be attributed to genetic factors, which are essential for selecting animals with superior milk-producing capabilities.

Table 1. Average milk production performances and heritability for the first two lactations in the Romanian Spotted Cattle Breed

COP	Lactation					
	Lactation I			Lactation II		
	n	$\bar{X} \pm S_{\bar{X}}$	h^2	n	$\bar{X} \pm S_{\bar{X}}$	h^2
1	862	21.12±0.198	0.314	910	24.14±0.237	0.297
2	908	21.18±0.206	0.272	1,014	23.25±0.224	0.253
3	914	20.81±0.202	0.236	1,036	22.27±0.228	0.222
4	947	19.91±0.199	0.254	1,061	21.18±0.22	0.237

COP	Lactation					
	Lactation I			Lactation II		
	n	$\bar{X} \pm S_{\bar{X}}$	h^2	n	$\bar{X} \pm S_{\bar{X}}$	h^2
5	997	18.93±0.189	0.194	1,070	20±0.205	0.184
6	1,003	18.38±0.186	0.251	1,035	19.29±0.212	0.241
7	976	17.7±0.189	0.24	1,055	18.02±0.195	0.225
8	980	17.02±0.181	0.238	1,053	17.17±0.189	0.228
9	900	16.37±0.196	0.304	958	16.25±0.192	0.287
10	748	15.97±0.214	0.381	792	15.38±0.204	0.372
Overall	9,235	18.76±0.195	0.265	9,984	19.76±0.210	0.251

Correlation between improvement values

The error variance values corresponding to the three biometric models considered are presented for different segments of the lactation period (1-5, 2-6, 3-7, 4-8, 5-9 and 6-10) in the first lactation (Table 2). From the results, it is evident that the lowest residual variances were obtained in the first lactation period (controls 1-5) using the P-Spline function, followed by the Legendre polynomial, and finally, the Natural Cubic Spline function. Specifically, the error variances in the order presented are 3.450, 3.989, and 5.421. These findings indicate that the P-Spline function is the most effective in capturing the underlying patterns of milk production during early lactation, while the Natural Cubic Spline function tends to produce higher residuals. Such differences in error variance are important for improving the accuracy of genetic evaluations and selecting models that best represent the lactation curve dynamics. Therefore, choosing the appropriate model can have significant implications for the efficiency of breeding programs in dairy cattle.

Table 2. Error variance associated with biometric models, depending on the serial number of controls, for lactation I

	Mathematical function	Order number of performed controls						
		1-5	2-6	3-7	4-8	5-9	6-10	1-10
Residual variance	Legendre polynomial	3.989	4.129	4.244	4.327	4.527	4.664	3.853
	P-Spline	3.450	3.582	3.856	3.879	4.093	4.459	3.329
	Natural Cubic Spline Function	5.421	5.507	5.732	5.706	5.421	6.209	5.172

The values obtained are consistent with previous findings, which indicate an inversely proportional relationship between the level of heritability and the size of the residual variance. Specifically, as heritability increases, residual variance tends to decrease, suggesting that higher genetic variability is associated with more precise estimations of milk production. To further explore this relationship, the degree of concordance between the improvement values calculated from 10 control points (considered as the control variant) and those derived from experimental variants, which used five performances taken sequentially throughout the lactation period, was assessed by calculating the correlation (Table 3).

Table 3. Correlation between the improvement values calculated between the control variant and the experimental variants for lactation I

	Mathematical function	Order number of performed controls						
		1-5	2-6	3-7	4-8	5-9	6-10	1-10
Correlation between improvement values	Legendre polynomial	0.890	0.884	0.881	0.878	0.872	0.860	1.00
	P-Spline	0.916	0.913	0.908	0.897	0.871	0.858	1.00
	Natural Cubic Spline Function	0.879	0.877	0.876	0.873	0.860	0.845	1.00

The results indicate that the lowest residual variances were obtained in the middle of the lactation period, specifically between the 4th and 8th test-day records. This suggests that mid-lactation provides the most reliable data for genetic evaluation, potentially offering a more accurate prediction of breeding values. These findings are important for refining breeding

programs, as they emphasize the importance of selecting appropriate test-day intervals to optimize the accuracy of genetic evaluations.
The 1-5 control interval was identified as the optimal variant, as it maximizes genetic progress by maintaining a high level of heritability. This interval also ensures a sufficiently strong concordance between the improvement values calculated from the control variant and those derived from the experimental variants. Specifically, the highest correlation was obtained using the P-Spline function (0.916), followed by the Legendre Polynomial (0.890), and the Natural Cubic Spline function (0.879).
These findings from the first lactation serve as a basis for evaluating similar trends in the second lactation, where comparable analyses will be conducted to assess the consistency of these results across different lactation periods.

Table 4. Error variance associated with biometric models, depending on the serial number of controls, for lactation II

	Mathematical function	Order number of performed controls						
		1-5	2-6	3-7	4-8	5-9	6-10	1-10
Residual variance	Legendre polynomial	4.023	4.229	4.441	4.848	5.440	7.235	3.981
	P-Spline	3.490	3.513	3.741	3.993	4.730	4.924	3.218
	Natural Cubic Spline Function	6.094	6.239	6.629	7.049	7.427	7.580	5.729

As shown in Table 4, the analysis of residual variance values for the second lactation reveals that, across the various segments of the lactation period (1-5, 2-6, 3-7, 4-8, 5-9, 6-10, 1-10), the smallest error variances were observed at the first part of the lactation period, specifically at controls 1-5. The P-spline function yielded the lowest variance, followed by the Legendre polynomial, and lastly, the Natural Cubic Spline function. The error variance values for these models were 3.490, 4.023, and 6.094, respectively. These results are consistent with previous studies, which have also demonstrated that mid-lactation provides the most accurate predictions for breeding value estimation.
With regard to the degree of concordance between the values calculated from the control variant and the experimental variants (Table 5).

Table 5. Correlation between the improvement values calculated between the control variant and the experimental variants for lactation II

	Mathematical function	Order number of performed controls						
		1-5	2-6	3-7	4-8	5-9	6-10	1-10
Correlation between the improvement values	Legendre polynomial	0.864	0.846	0.842	0.839	0.827	0.773	1.00
	P-Spline	0.878	0.885	0.871	0.839	0.833	0.778	1,00
	Natural Cubic Spline Function	0.842	0.839	0.833	0.830	0.813	0.803	1.00

It is observed that the highest correlations were achieved for controls 1-5, with the P-Spline function yielding the highest value (0.878), followed by the Legendre polynomial (0.864), and lastly, the Natural Cubic Spline function (0.842). These findings suggest that the performances recorded during this period provide a more accurate estimate of the improvement value, reinforcing the results obtained in the first lactation. Such consistency across both lactation periods highlights the reliability of the selected control intervals for genetic evaluation. These results are consistent with similar studies by other authors (White et al., 1999; Macciotta, 2005; 2008; 2011; Silvestre, 2005; 2010; Misztal, 2006), who also found that mid-lactation controls offer the best predictive power for breeding value estimation.

CONCLUSIONS

The findings of this study emphasize that modeling the lactation curve using advanced biometric functions significantly enhances the accuracy of estimating the improvement value in Romanian Spotted Cattle. Among the three

models analyzed (Legendre Polynomial, P-Spline, and Natural Cubic Spline), the P-Spline function demonstrated superior adjustment capacity, yielding the lowest error variances and the highest correlations between the improvement values estimated based on the 5-control variants and the control variant (10 controls).

The results further indicated that the heritability of milk production is highest in the early part of lactation (controls 1-5), which facilitates more precise genetic evaluation. Consequently, reducing the number of measurements from 10 to 5 does not compromise prediction accuracy but rather optimizes the genetic selection process by reducing the costs and resources associated with performance control.

Based on these findings, it is recommended that, in predicting the breeding value of Romanian Spotted Cattle, the P-Spline function be employed using performances measured between controls 1-5, as per the A4 method. This approach can substantially contribute to optimizing genetic progress while lowering the costs associated with conducting official performance control (COP).

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GENETIC INSIGHTS FROM NEUTRAL AND ADAPTIVE MARKERS GUIDE CONSERVATION OF LESSER KESTREL POPULATIONS IN BULGARIA

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Abstract

The genetic diversity and population structure of the Lesser Kestrel (Falco naumanni), a threatened migratory falcon, were assessed to inform conservation strategies under the LIFE project for the species' reinforcement in Bulgaria, by means of neutral microsatellite markers and non-neutral candidate gene markers associated with migratory behavior. The Core European populations (Spain, Italy, Greece) exhibit high genetic diversity and low differentiation, making them suitable sources for conservation translocations. In contrast, peripheral populations (e.g., Mongolia, Limnos Island) are genetically distinct and less suited for reinforcement due to potential risks of outbreeding depression. Non-neutral markers showed minimal genetic differentiation among populations, suggesting a lack of disruptive adaptive divergence within the species' range. These findings emphasize the importance of genetic similarity and diversity in translocation strategies and highlight the potential for maintaining adaptive potential while avoiding maladaptive outcomes. This integrated approach offers a framework for enhancing the genetic resilience and long-term stability of Lesser Kestrel populations in Bulgaria.

Key words*: conservation translocation, genetic diversity, genetic management, microsatellite markers, population structure.*

INTRODUCTION

Genetic differentiation and gene flow patterns in several species are shaped by the ecological features of their habitats and whether they migrate or not (Willoughby et al., 2017). Birds can exhibit natal and breeding-site fidelity in addition to long-distance migratory behavior, especially raptors (Grande et al., 2009; Newton, 2010). In general, migratory raptor populations show higher genetic diversity compared to non-migratory populations; however, they tend to show low levels of genetic differentiation among geographically isolated groups, resulting in a weak population structure. but at the same time population structure is weak (Webster et al., 2002). However, fine-scale genetic structure can be seen in demographically decreased populations because of the fidelity to the natal and breeding sites (Bounas et al., 2017; Di Maggio et al., 2015). Moreover, populations that are regionally isolated and patchily distributed, are more vulnerable to genetic drift, which will ultimately drive a population to genetic diversity loss. Additionally, a population that suffers from inbreeding may further exhibit lower fitness. This would increase species' risk of extinction by impairing its capacity to adapt to a changing environment (Amos & Balmford, 2001; Frankham, 1996; Reed & Frankham, 2003). Therefore, knowing a species' genetic diversity, population structure, and the processes that shape them is one of the most crucial things that need to be considered in order to inform a reintroduction strategy.

A species of particular conservation concern where such genetic considerations are critical is the Lesser Kestrel (*Falco naumanni*). This migratory falcon exhibits broad distribution, breeding in higher latitude from the Mediterranean across to Mongolia and China, and its wintering grounds are located in Sub-Saharan Africa (Cramp & Simmons, 1980). The species went through severe declines across its distribution, at least in Europe in the early ‘60s, mainly due to intensification of agriculture and

subsequent habitat degradation along with changes in land use (Inigo & Barov, 2010). Particularly in the Central and Eastern Mediterranean region the decrease was severe, resulting in local extinctions and thus causing range reduction and population fragmentation (Inigo & Barov, 2010). Today, the species still has a "patchy" distribution despite the fact that its extincrion risk is low (BirdLife International, 2017). However, because of the recent recolonization of several sites across its European distribution, it has been downlisted to 'Least Concern' by IUCN. Despite the encouraging signals of population recovery, still its abundance is low compared to the one described for the '60s. Therefore, actions that will foster the recovery and the stabilization of populations both in the core and the expanding peripheral populations are still needed.

Genetic management is particularly critical in conservation translocations, aiming to enhance demographic stability while addressing the challenges of maintaining genetic diversity and adaptive potential of populations. Translocating raptors to peripheral or newly established colonies can serve as an effective conservation action that may lead to short-term population stabilization or even growth (Morandini & Ferrer, 2017; Seddon et al., 2014). Nonetheless, these programs should aim at enhancing both the genetic and the adaptive similarity between native and introduced populations to reduce any adverse adaptive and evolutionary outcomes (Holderegger et al., 2006). Thus, a central aim of conservation translocation programs should be the preservation of both demographic stability and genetic variation. Off-site conservation breeding serves three main functions in a management or recovery program aimed at preserving a specific species: a) to offer demographic and/or genetic support for current wild populations, b) to create sources for founding new populations in the wild, and c) to prevent extinction of populations that lack an immediate chance of survival in the wild by keeping them in captivity (Allendorf & Luikart, 2009). Choosing the initial pool of the founding individuals for a captive breeding programme is a crucial and challenging task for many species. Individuals selected to provide demographic and genetic assistance for wild populations should be chosen to enhance genetic and ecological (adaptive) diversity. In contrast, newly introduced populations must possess sufficient genetic diversity to adapt to their new environment.

The majority of research regarding the genetic effects of translocations examines diversity at putatively neutral markers like microsatellites, revealing population structure and differentiation patterns influenced by neutral evolutionary forces, such as genetic drift, mutation, and gene flow or dispersal (Kirk & Freeland, 2011). Nonetheless, genetic variation encompasses another component that is the functional diversity; populations under the influence of different environmental factors may have evolved in response to different selective pressures. Migratory birds, such as the Egyptian Vulture, exhibit complex behavioral and physiological responses that are likely influenced by genetics and could be shaped by natural selection (Liedvogel et al., 2011; Pulido, 2007). A few candidate gene markers (e.g., ADCYAP1, CREB1, CLOCK and NPAS2), have been identified by analysing circadian molecular pathways in the avian genome for tandem repeats (Steinmeyer et al., 2009). Examining variations in these genes could provide fresh perspectives on how populations adapt locally and the evolutionary and ecological consequences of this adaptation.

This paper provides genetic management recommendations to inform strategies that will preserve the allelic diversity of the Lesser Kestrel and strengthen the Bulgarian population of the species, after its nesting was restored in the country by Green Balkans in 2014 (Gradev et al., 2016). To address this aim, this study provides an assessment of the genetic diversity levels and genetic structure of the Lesser Kestrel populations across most of the range of the species'. Specifically, we examine both neutral markers, which help assess overall genetic diversity and reveal patterns shaped by random processes like gene flow and genetic drift, and adaptive markers, which offer insights into how populations may respond to environmental pressures and selective forces. This distinction is crucial for understanding both the current genetic health of populations and their potential for long-term survival and adaptation. Then, the assessment of genetic similarity, the preservation of traits suited to local

environments, will help pinpoint the most suitable source populations that can be used for reinforcement actions thus safeguarding the Bulgarian population's genetic diversity, given that the population is still in low numbers, with 40-50 breeding pairs distributed just in 3-4 colonies (Gradev et al., 2021). Given these low numbers, this study aims to provide genetic insights to inform conservation strategies that can bolster the population's genetic diversity and contribute to its long-term survival.

MATERIALS AND METHODS

We used two genetic datasets that utilise samples from 15 sites throughout the species distribution, from the Iberian Peninsula to Mongolia (Figure 1): A set of 295 individual genotypes for 18 microsatellite loci after Bounas et al. (2017) and a set of 96 genotypes for repeat-rich regions within ADCYAP1, CREB1, CLOCK, and NPAS2 genes (Steinmeyer et al., 2009).

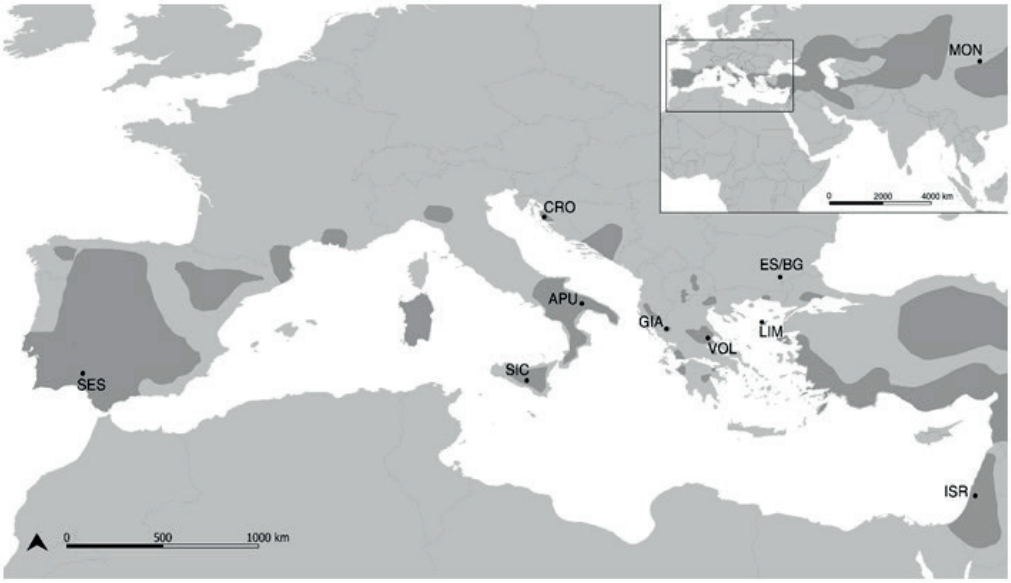


Figure 1. Map of the lesser kestrel populations used in the present study. SES: Andalucia, ES/BG: Bulgaria (Extremaduran origin), APU: Apulia, SIC: Sicily, CRO: Croatia, GIA: Ioannina, LES: Agrinio, TRI: Trikala, LAR: Larisa, VOL: Volos, KIL: Kilkis, KAL: Komotini, LIM: Limnos, ISR: Israel, MON: Mongolia. Shaded areas represent the breeding distribution of the species (modified from BirdLife International)

The four candidate genes were amplified in a single multiplex reaction (Chakarov et al., 2013) using the KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems). In each reaction, we inserted 20 ng template DNA, 2 pM of each primer and 1 × KAPA2G Mix.

The PCR settings were the following: first, an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 15 s at 95°C, then an annealing step of 30 s at 60°C and a final extension step of 30 s at 72°C, with a final extension step of 10 min at 72°C.

PCR products were run on an ABI 3730xl capillary sequencer (Applied Biosystems) and final genotypic data were obtained using STRand v.2.4.59 (Toonen & Hughes, 2001).

To assess the genetic diversity in each population we calculated standard genetic diversity indices including the number of alleles, heterozygosities (observed and expected) and private allele richness. For this we used GENALEX v.6.5 (Peakall & Smouse, 2012) and HP-RARE (Kalinowski, 2005). We used FSTAT 2.9.3.2 (Goudet, 2002) to calculate allele richness, corrected for population sample size, and further calculated inbreeding coefficient (Fis), tested for deviations from Hardy-Weinberg (HW) proportions at locus and population levels, and finally test loci for linkage disequilibrium (LD). For these we used 1000 randomizations and adjusted the significance for multiple comparisons.

To describe the pattern of genetic structure across the datasets, first we computed pairwise fixation indices (Fst) among populations and estimated 95% confidence intervals (1000 bootstraps) in "hierfstat". Jost's D was also calculated among populations using the package "mmod" (Winter, 2012) in R. In addition, we performed a principal component analysis (PCA) using "adegenet" (Jombart, 2008). Population structure was additionally assessed by subjecting all individuals in a Bayesian clustering analysis implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). This analysis allowed to infer the number of genetically homogeneous clusters (K) present in both the neutral and the non-neutral datasets. We used the admixture ancestry model and correlated allele frequencies (Falush et al., 2003), and runs were set with a burn-in period of 10^5 iterations followed by 5×10^5 MCMC steps with 10 replicates for each K value (1-9). Structure results were then further analysed to identify the number of genetic clusters formed by the sampled individuals, using the ΔK method (Evanno et al., 2005) from STRUCTURE HARVESTER (Earl & Vonholdt, 2012). Furthermore we calculated the posterior probability for each K. Finally isolation by distance patterns were explored by conducting a Mantel test between pairwise genetic (Fst/1 − Fst) and geographic distance matrices using the R package "ade4" (Dray & Dufour, 2007).

RESULTS AND DISCUSSIONS

Results on the populatin genetic diversity, i.e. number of alleles, observed and expected heterozygosity, allelic richness, private alleles and inbreeding coefficient, based on the neutral loci are shown in Table 1.

Table 1. Measures of genetic variation of all sampled lesser kestrel populations based on microsatellite markers

Location	Code	n	A	Ar	Ho	He	π	Fis
Croatia	CRO	14	5.625 (0.645)	4.7 (0.45)	0.654 (0.048)	0.65 (0.048)	0	-0.023 (0.043)
Bulgaria (Extremaduran, Spain origin)	ES/BG	25	8.250 (1.289)	5.6 (0.66)	0.674 (0.040)	0.699 (0.037)	6	0.029 (0.037)
Andalucia, Spain	SES	19	7.125 (0.930)	5.4 (0.55)	0.635 (0.037)	0.696 (0.037)	1	0.082 (0.041)
Central Greece	VOL	20	7.563 (1.194)	5.5 (0.68)	0.678 (0.051)	0.662 (0.048)	1	-0.027 (0.035)
Ioannina, Greece	GIA	24	7.563 (1.099)	5.2 (0.56)	0.634 (0.051)	0.654 (0.048)	2	0.036 (0.031)
Limnos, Greece	LIM	11	5.125 (0.562)	4.8 (0.49)	0.661 (0.060)	0.667 (0.041)	1	0.024 (0.060)
Israel	ISR	20	7.250 (1.097)	5.5 (0.66)	0.619 (0.062)	0.682 (0.042)	5	0.124 (0.055)
South Italy	APU	44	9.375 (1.463)	5.5 (0.55)	0.669 (0.038)	0.707 (0.036)	3	0.053 (0.025)
North Mongolia	MON	17	7.188 (0.963)	5.4 (0.59)	0.585 (0.057)	0.655 (0.049)	10	0.155 (0.046)
Sicily	SIC	12	5.938 (0.814)	5.1 (0.615)	0.682 (0.038)	0.667 (0.041)	2	-0.045 (0.045)

Note: number of sampled individuals (n), number of alleles (A), allelic richness (Ar), observed (Ho) and expected (He) heterozygosity (along with their respective standard errors, SE), rarefied private allelic richness (π) and inbreeding coefficient (Fis) along with 95% confidence intervals (CI).

Allelic richness was highest in the core European populations of Spain Greece and Italy but low in the Croatian and Limnos island populations (LIM, CRO). The highest heterozygosity values were observed in the Sicilian (SIC) population whereas the lowest levels were found in the Mongolian population (MON).

Regarding the putatively adaptive loci, all four genes showed low levels of genetic diversity with four, three, two and one alleles detected in ADCYAP1, CREB1, CLOCK and NPAS2 respectively (Table 2).

Table 2. Estimates of genetic diversity of the sampled Lesser Kestrel populations based on the candidate gene data

Population	locus	n	Na	Ho	He	π	Fis
ES/BG	*Adcyap1*	15	4	0.40	0.38	-	0.012
	Creb1	15	2	0.20	0.28	-	0.311
	Clock	15	1	0.00	0.00	-	-
	Npas2	15	2	0.07	0.06	-	0.000
APU	*Adcyap1*	15	3	0.47	0.44	-	-0.032
	Creb1	15	2	0.27	0.44	-	0.429
	Clock	15	2	0.13	0.12	-	-0.037
	Npas2	15	2	0.07	0.06	-	0
CRO	*Adcyap1*	12	3	0.42	0.50	2	0.203
	Creb1	12	3	0.33	0.56	-	**0.439**
	Clock	9	2	0.11	0.10	-	0.000
	Npas2	12	1	0.00	0.00	-	-
ISR	*Adcyap1*	15	3	0.53	0.58	-	0.122
	Creb1	15	2	0.40	0.44	-	0.134
	Clock	15	2	0.13	0.12	-	-0.037
	Npas2	15	1	0.00	0.00	-	-
LIM	*Adcyap1*	9	2	0.56	0.40	-	-0.333
	Creb1	3	2	1.00	0.50	-	-1.000
	Clock	5	1	0.00	0.00	-	-
	Npas2	10	1	0.00	0.00	-	-
MON	*Adcyap1*	14	2	0.36	0.44	-	0.217
	Creb1	14	2	0.14	0.34	-	**0.600**
	Clock	14	2	0.07	0.07	-	0.000
	Npas2	14	2	0.07	0.07	-	0.000
CGR	*Adcyap1*	15	3	0.60	0.53	-	-0.105
	Creb1	14	2	0.21	0.50	-	**0.594**
	Clock	14	2	0.07	0.07	-	0.000
	Npas2	15	2	0.07	0.06	-	0.000

Note: number of sampled individuals (n), Number of alleles (A), observed (Ho) and expected (He) heterozygosity and inbreeding coefficient (Fis). Statistically significant values are written in bold.

Observed heterozygosity for adaptive loci was generally low except for ADCYAP1 gene. For this gene, Central Greece population showed the highest heterozygosity levels (Ho = 0.60). Populations did not show any significant differences between observed and expected heterozygosity in ADCYAP1, CLOCK and NPAS2, but in locus CREB1, observed heterozygosity was significantly lower than expected in Central Greece, Mongolia and Croatia.

According to the neutral dataset, STRUCTURE results some population structure. When modelling all individuals, the ΔK-method indicated that two clusters (K = 2) represented the most likely population structure. In this scenario, all European populations were found to belong in the first cluster, and the MON and LIM populations exhibited high membership to the second (Figure 2). Lesser kestrels from Israel (ISR) were determined to be significantly admixed (Figure 2). In the scenario of K = 3, a third gene pool is mostly represented by the Croatian population. There were no signs of any further substructure in the core European populations of Spain, Italy and Central Greece (Figure 2). When the model was run for the non-neutral dataset, the posterior probability value suggested K = 1, suggesting there is no structure among populations.

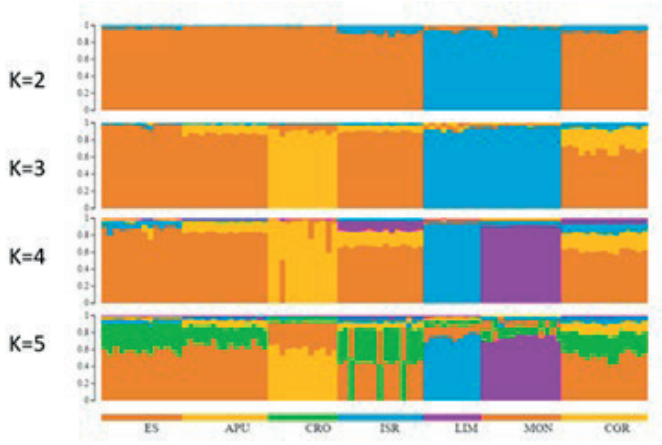


Figure 2. Admixture proportions (proportions of membership to each of K inferred clusters) of individual lesser kestrels for the neutral dataset (own source)

Similarly, Fst and D values among populations, did not show any statistically significant differences based on the non-neutral dataset. Using the microsatellite dataset, Fst values largely confirmed STRUCTURE results (Table 3). Finally, genetic distance did not show any association with geographical distance across all populations ($r^2 = 0.05$, $p = 0.9$).

Table 3. Pairwise Fst-values (below diagonal) and D values (above diagonal) among Lesser Kestrel populations based on the neutral microsatellite markers

	ES/BG	**APU**	**CRO**	**ISR**	**LIM**	**MON**	**CGR**
ES/BG	-	0..00	**0..04**	**0.02**	**0.03**	**0.04**	0.01
APU	0.00	-	0.03	0.01	**0.03**	**0.04**	0.01
CRO	**0.08**	**0.06**	-	**0.02**	**0.07**	**0.07**	0.01
ISR	**0.04**	0.02	**0.05**	-	**0.03**	**0.04**	0.01
LIM	**0.07**	**0.08**	**0.14**	**0.08**	-	**0.04**	**0.05**
MON	**0.09**	**0.10**	**0.14**	**0.08**	**0.08**	-	**0.05**
CGR	0.01	0.01	**0.03**	0.03	**0.10**	**0.10**	-

The analysis of neutral microsatellite data revealed that the core European populations - specifically those from Spain (already comprising the founder Bulgarian population), Greece, and Italy (APU) - exhibited the highest levels of allelic richness and genetic diversity. These populations also showed low genetic differentiation among each other, suggesting a high degree of gene flow and a shared genetic background. In contrast, the Croatian (CRO) and Limnos Island (LIM) populations displayed the lowest allelic richness and higher levels of genetic differentiation. The Mongolian population was also genetically distinct from the European populations, as indicated by the Bayesian clustering analysis and pairwise Fst values.

The lack of significant genetic structure among the core European populations implies that they constitute a relatively homogeneous genetic group. This homogeneity, coupled with their high genetic diversity, makes them suitable candidates for sourcing individuals for reinforcement efforts in Bulgaria. The genetic differentiation of the Mongolian and Limnos populations suggests that translocating individuals from these populations may not be advisable due to potential genetic incompatibilities and the risk of outbreeding depression.

The analysis of the putatively adaptive candidate genes (ADCYAP1, CREB1, CLOCK, and NPAS2) did not reveal significant genetic differentiation among populations. The low levels of genetic diversity and lack of structure in these genes may indicate that selective pressures on migratory behavior are similar across the species' range, or that these markers are not sufficiently variable to detect adaptive differences. This finding suggests that, from the perspective of the candidate genes studied, there may be minimal risk of disrupting local adaptations through translocations among European populations.

The absence of a significant isolation-by-distance pattern further supports the notion that geographic distance is not a major barrier to gene flow among Lesser Kestrel populations in Europe. This is consistent with the species' migratory behavior and capacity for long-distance dispersal, which can facilitate genetic exchange across broad spatial scales.

CONCLUSIONS

The present report provides a comprehensive assessment of the genetic diversity and population structure of Lesser Kestrel populations across a substantial portion of the species' range, utilizing both neutral microsatellite markers and putatively adaptive candidate genes associated with migratory behavior. The findings offer critical insights into the genetic landscape of the species, which are essential for informing conservation strategies aimed at reinforcing the Bulgarian population and preserving the overall genetic diversity of the species.

Given the current genetic composition of the Bulgarian population (founders of spanish origin) and the findings of this study, sourcing additional individuals from other genetically diverse core populations, such as Greece and Italy, could enhance the genetic diversity and adaptive potential of the Bulgarian population. Incorporating individuals from these populations may introduce novel alleles and promote heterozygosity, thereby strengthening the population's resilience to environmental changes and reducing the risks associated with inbreeding. Such an approach aligns with conservation best practices that advocate for maximizing genetic similarity and minimizing the introduction of maladaptive alleles.

However, caution is needed when considering translocations from genetically distinct populations, such as those from Mongolia or Limnos Island. Introducing individuals from these populations could lead to outbreeding depression if local adaptations are disrupted. Therefore, translocation efforts should prioritize populations that are genetically similar to the target population.

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EFFECT OF ABCG2 GENE POLYMORPHISM ON MILK PRODUCTIVITY IN AWASSI EWES

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Abstract

The ABCG2 gene is responsible for transporting many molecules across cell membranes and is expressed in various tissues, including the mammary gland. In this regard, the ABCG2 gene has been considered as a candidate gene related to the quantity, composition and quality of milk yield in sheep. The purpuse of present study was to establish the genetic diversity in this gene in connection with the study of possible dependencies of genotypes in ABCG2 with milk production in the ewes of the Awassi breed reared in Bulgaria. A highly polymorphic state of the ABCG2 gene was found, with the presence of two alleles - wild "+" and mutant "-" with frequencies of 0.49 and 0.51, respectively, and three genotypes - homozygous +/+ (0.31), heterozygous +/- (0.36) and homozygous -/- (0.33). Regarding milk productivity, no statistically significant differences were found between the different genotypes of the ABCG2 gene.

Key words*: Awassi sheep, ABCG2 gene, PCR-RFLP, genetic polymorphism, milk productivity.*

INTRODUCTION

Sheep farming is a potentially profitable agricultural activity in areas unsuitable for other agricultural production. Sheep milk, which is more suitable for processing than cow and goat milk, is obtained predominantly from low- to medium-yielding local breeds raised under extensive conditions for meat, milk, wool and hides (Gutiérrez-Gil et al., 2014), but also from specialized dairy breeds. Globally, the demand for sheep milk and its products, such as cheese and yogurt, is influenced by consumer preferences for traditional and specialty dairy products. As the world population continues to grow, the consumption of a variety of dairy products is expected to increase, highlighting the importance of sustainable development in the sheep milk sector. To meet growing consumer demands, it is necessary to increase sheep milk yield and improve the efficiency of sheep farming practices (Garzón et al., 2023; Ceyhan et al., 2022).

The Food and Agriculture Organization of the United Nations (FAO) forecasts that world milk production will reach approximately 979 million tons in 2024, reflecting an increase of 1.4% compared to 2023. Milk production takes place in all EU countries and represents a significant share of the value of EU agricultural output. Total EU milk production is estimated at around 155 million tons per year according to Eurostat. While cow's milk dominates the market, accounting for around 81% of total production, sheep's milk, together with goat's and camel's milk, contributes around 4%. This suggests that although sheep's milk represents a smaller share of the global dairy sector, it remains an important and specialised component of the industry. Most sheep's milk production is concentrated in certain regions, particularly in the Mediterranean countries and the Balkans, where sheep farming has deep-rooted cultural and economic importance. In the European Union, countries such as Spain, Greece, France and Italy are notable producers of sheep milk, with Spain producing approximately 1.1 million tons and Greece 0.9 million tons from sheep and goats combined in 2023 (Mioč et al., 2024; FAO, 2024).

In Bulgaria, sheep milk production has remained relatively stable in recent years. According to data from the Ministry of Agriculture and Food, in 2022, sheep milk production in Bulgaria amounted to 54.742 million litres, which represents a significant decrease of 19.5% compared to 2021. The dairy industry in Bulgaria is experiencing structural changes and market consolidation, which contributed to increasing

efficiency in dairy farming and milk processing. In 2023, a total of 50,255 thousand litres of raw milk were produced in Bulgaria from 901,200 ewes. As of November 1, 2023, the total number of sheep in the country was 1,072,768 - 2.2% below the level of a year ago (Agricultural Report, 2024).

Genomic markers for genetic variation allow selective intervention in breeding and early assessment with high accuracy, so that good production traits in sheep can be better transmitted to their offspring (Hayes et al., 2013; Wang et al., 2023). In recent years, various candidate genes related to yield and milk production qualities have been tested, such as DGAT1, β-lactoglobulin, prolactin, ATP-binding cassette subfamily G member 2 (ABCG2) (Gutiérrez-Gil et al., 2014; Xu & Li, 2017; Singh et al., 2019).

ABCG2 is an ABC transporter, a member of the ATP-binding cassette (ABC) superfamily, which is thought to be important for protection against xenobiotics (chemical compounds that do not belong to the natural composition of living organisms) (Robey et al., 2009). ABCG2 also has an important function in the development and differentiation of the mammary gland (Alim et al., 2013). The gene encoding the ABCG2 protein is located on chromosome 6 of the ovine genome, is composed of 20 exons separated by introns, and is expressed in several tissues, including the mammary gland (Al-Mamun et al., 2015).

ABCG2 is thought to be associated with milk production traits in sheep (Árnyasi et al., 2013), for example in the Manech Tête Rousse, a dairy breed used for cheese production in the Pyrenees region (Rochus et al., 2018). According to Gutiérrez-Gil et al. (2014) the ABCG2 gene strongly influences the amount of milk produced in sheep. Studies have shown the presence of a polymorphism in the ABCG2 gene. A mutation representing an insertion/deletion of 35 bp was found in the ABCG2 gene of sheep (Árnyasi et al., 2013). Oner et al. (2014) reported the "-" allele as the predominant allele and its frequency was in the range of 0.50-0.65. In a study by Árnyasi et al. (2013) the "+" allele was observed with a higher frequency, establishing a relationship between the "-" allele and the higher SCC in the milk of the studied sheep.

Hofmannová et al. (2018) identified a polymorphism in intron 5 of the ABCG2 gene in the Lacaune and East Friesian breeds raised in the Czech Republic. This mutation is located in the non-coding region and does not cause changes in the amino acid sequence, but the introns may carry transcriptional regulatory elements (Árnyasi et al., 2013; Hofmannová et al., 2018). In Lacaune animals, the deletion allele is predominantly found, while in East Friesian animals the non-deletion allele predominates. This mutation of the ABCG2 gene had an effect on the somatic cell count in milk. The I allele may be associated mainly with the lower limit of the somatic cell count (SCC) in the milk of healthy animals, for which increased resistance to mastitis has not been demonstrated (Hofmannová et al., 2018).

Climate change both globally and in our latitudes is one of the reasons for the search for more resistant sheep breeds. Awassi is the most numerous and widespread sheep breed in southwest Asia, with variation observed between individual flocks in terms of milk productivity, size and wool quality (Epstein, 1985). The milk yield of Awassi sheep varies widely (from 73 kg to 506 kg) (Galal et al., 2008; Al-Samarai & Al-Anbari, 2009; Gootwine, 2011; Meydan et al., 2024) due to various reasons: level of selection, duration of lactation, climatic influences, birth order, milking frequency, production year and last but not least genetic diversity (Ali et al., 2020; Meydan et al., 2024). The interest in the Awassi breed is also related to healthy eating - the milk fat of the Awassi breed has a better ratio of omega-3 and omega-6 compared to the milk fat of other breeds (Ayadi et al., 2024), and in general we can say that animals of the Awassi breed have higher quality meat and milk (Merzah et al., 2023).

The present study aims to determine the genetic diversity in intron 5 of the ABCG2 gene in connection with the study of possible dependencies of genotypes in ABCG2 with milk production in the ewes of the Awasi breed reared in Bulgaria.

MATERIALS AND METHODS

Animals. The study was conducted with ewes of the Awassi breed, raised in a private flock in the village of Sredkovets, Shumen district, Bulgaria (Figure 1). The flock was also the subject of our previous study (Dimitrova et al., 2024) with the consent of the owner. Blood samples were collected from the jugular vein of 61 clinically healthy animals in vacuum tubes containing EDTA as an anticoagulant by the state veterinarian in charge of the farm.



Figure 1. Awassi ewes from a private herd - the village of Sredkovets, Shumen district, Bulgaria (own source)

The animals are raised in a semi-intensive, pasture-manure system. The lambing campaign on the farm usually starts in the second half of December and ends by the end of March, according to the insemination campaign in the respective year. Milking is mechanized and is carried out twice, after the lambs are weaned at about 2-3 months of age.

The control of the productive qualities of the Awassi sheep breed in Bulgaria is carried out by the "Breeding Association for Dairy Sheep" (RAMO). According to the regulatory provisions, control of milk productivity was carried out by measuring the amount of milk in liters milked during the milking period of the animals (1st and 2nd lactation) using the AC method, specified in the nomenclature of the International Committee for Animal Control (ICAR). For the studied herd, the provided data on the individual milk productivity of the controlled sheep were equated for a 120-day milking period (TMM120).

DNA isolation Genomic DNA was extracted from whole blood using a manual commercial DNA purification kit (Illustra Blood Genomic Prep DNA Purification Kit, GE Healthcare) following the manufacturer's protocol. The quality and quantity of the DNA were assessed via spectrophotometric analysis (Biodrop spectrophotometer) and agarose gel electrophoresis using a 1% agarose gel (Bioline) with 1× TAE buffer (Thermo Scientific).

PCR procedure PCR reactions were conducted in a total volume of 10 µL, containing 40 ng of DNA template, 0.2 µL ddH_2O, 20 pM of each primer, and 5 µL of 2× (1.5 mM $MgCl_2$) MyTaq™ HS Red Mix (Bioline).

The primer set used for amplification (F:5'-GCCTCTTCTCCCATACGTC-3' and R:5'-AAACCAGTTGTGGGCTCATC-3') was based on the study by Árnyasi et al. (2013), generating PCR products of 232 bp (with deletion) or 267 bp (no deletion).

PCR amplifications were performed using a erityPro 96-Well (Applied Biosystems by Thermo Fisher Scientific) according to the following parameters: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (52.6°C for 65 s), and elongation (72°C for 1 min), with a final extension step at 72°C for 10 min.

The fragment sizes were determined by agarose gel electrophoresis, using a Ready-to-Use 50 bp DNA Ladder (Thermo Scientific) on a 2.4% agarose gel (Bioline), stained with RedGel™ Nucleic Acid Stain (Biotium), and analyzed under UV exposure.

Statistical analysis. The data on milk yield for a 120-day milking period (TMM120) were processed using the Statistica software product. To determine the effect of the ABCG2 gene polymorphism on milk yield for a 120-day milking period, the one-way ANOVA analysis of variance model was used.

RESULTS AND DISCUSSIONS

Genotyping of the ABCG2 gene was performed by polymerase chain reaction (PCR) itself, capable of distinguishing between fragments based on the presence or absence of the mutation (deletion/insertion) and subsequent electrophoresis. As a result, fragments of intron 5 of the ABCG2 locus with two different lengths were amplified - 232 bp resulting from the deletion and 267 bp in the absence of the deletion (Figure 2).

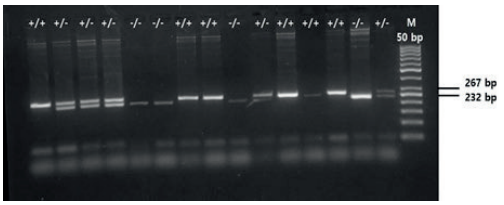


Figure 2. Agarose gel electrophoresis of PCR fragments of ABCG2 gene and DNA ladder visualized on 2.4 % agarose gel under UV light (own source)

The fragment representing the wild-type allele "+" is 267 bp in size, and the fragment with a 35 bp deletion representing the mutant allele "-" is 232 bp in size. A highly polymorphic state of the ABCG2 gene was found in Awassi sheep, the wild-type allele "+" has a frequency of 0.49, and the mutant allele "-" - 0.51. Three genotypes were identified with the following frequencies - homozygous wild type +/+ - with 0.31, heterozygous +/- with 0.36 and homozygous mutant -/- - with 0.33. In this herd, however, the expected heterozygosity is higher than the established one, and the inbreeding coefficient is positive, which also indicates the presence of inbreeding, and the results obtained are statistically reliable (Table 1).

Table 1. Allele and genotype frequencies, observed and expected heterozygosity, coefficient of inbreeding in studied Awassi herd

Breed	n	Allele frequency		Genotype frequency			Heterozygosity		F_{is}	χ^2	p
		+	-	+/+	+/-	-/-	Ho	He			
Awassi ABCG2	61	0.49	0.51	0.31	0.36	0.33	0.361	0.500	0.278	5.39	0.02*

*statistically significant difference ($p<0.05$)

In our previous study of the ABCG2 gene in Bulgarian sheep breeds, we tested a total of 90 animals from three Merino breeds - Askanian Merino, Caucasian Merino and Karnobat Merino, and polymorphism was found in all three breeds with the presence of two alleles and three genotypes, with the mutant allele frequencies being higher. The frequency of allele "-" was highest in Askanian Merino sheep breeds - 0.76 and the frequency of genotype -/- is also highest in animals of the same breed - 0.68 (Dimitrova et al., 2019).

A predominant allele "–" was reported by Oner et al. (2014) in Turkish sheep breed Kıvırcık and frequency was in the range of 0.60. On the contrary, the "+" allele was found with a higher frequency in another study conducted in Hungary in Gyimesi Racka and Awassi sheep (Árnyasi et al., 2013). The genotype frequencies for the Awassi breed were: 0.39 for +/+, 0.46 for +/- and 0.15 for -/-, which differ from the results obtained by us for the same breed.

Hofmannová et al. (2018) identified all three genotypes in a study of Lacaune and East Frisian sheep. In the Lacaune breed, the deletion allele was predominantly found (0.694), while in the East Frisian the non-deletion allele was primarily found (0.784).

Bozhilova-Sakova et al. (2022) studied two flocks of the Bulgarian dairy synthetic population - the first from the Agricultural Institute in Shumen - 116 ewes with frequencies of the wild-type allele "+" - 0.53 and of the mutant allele "-" 0.47; the second from the Institute of Animal Husbandry - Kostinbrod - 68 ewes with frequencies of the wild-type allele "+" - 0.71 and of the mutant allele "-" - 0.29. All three possible genotypes were identified in both flocks. In the sheep from Shumen, the heterozygous genotype had a frequency of 0.63, and in those from Kostinbrod - 0.32; both flocks were not in Hardy-Weinberg equilibrium. The results obtained for both herds differ significantly from our results.

In our previous study, with sheep from the same flock, we found that the total milk productivity of the sheep was 126.317 (l) per 120-day milking period (Dimitrova et al., 2024). It is known that the milk productivity of the Awassi breed sheep varies in different countries of its distribution (Galal et al., 2008). For example, Üstüner & Mustafa (2013) found that the milk yield of the Awassi breed sheep in the Central Anatolian Region of Turkey was 196.5 kg for a 184.3-day lactation period, and for sheep of the same breed in Iraq, Al-Samarai et al., (2014) reported a milk yield of 103.57 kg for a 107.44-day lactation period. The milk productivity of the genotypes identified in the present study is shown in Table 2.

Table 2. Effect of the genotype of ABCG2 gene on the milk yield for a standard 120-day milking period

Traits	Genotype +/+ (n=19)			Genotype +/- (n=22)			Genotype -/- (n=20)			p-value
	Number of milk records	Average	CV%	Number of milk records	Average	CV%	Number of milk records	Average	CV%	
TMM120, total	28	126.123	45.90	35	126.645	38.15	38	126.158	46.89	0.94

The carriers of the three genotypes are almost evenly distributed, their milk yield is close to the average for the studied sheep, and is in very close limits for the homozygous genotypes "+/+" (126.123 l) and "-/-" (126.158 l). The milk yield is slightly higher for the heterozygous genotype "+/-" (126.645 l), but no relationship between the ABCG2 gene polymorphism and milk yield in the different genotypes was established.

In a study conducted on 30 sheep from the Bulgarian Dairy Synthetic Population, two genotypes were identified in intron 5 of the ABCG2 gene: homozygous (-/-) and heterozygous (+/-). The analysis revealed no statistically significant difference in milk production between heterozygous individuals (+/-) and those with the homozygous (-/-) genotype at a 5% significance level, corroborating the findings of the present study (Bozhilova-Sakova et al., 2022a).

CONCLUSIONS

A genetic polymorphism in intron 5 of the ABCG2 gene was identified in the studied Awassi ewes with the presence of two alleles with similar frequencies. Three genotypes were identified with the following distribution - homozygous wild type +/+ - with 31%, heterozygous +/- - with 36% and homozygous mutant -/- - with 33%. The expected heterozygosity is higher than the established one, and the inbreeding coefficient is positive, which indicates the presence of inbreeding in the studied herd of the Awassi breed.

The milk yield of ewes carrying the three genotypes is nearly identical, closely aligning with the average yield of the studied sheep. The homozygous genotypes "+/+" (126.123 l) and "-/-" (126.158 l) exhibit very similar yields, while the heterozygous genotype "+/-" shows a slight increase (126.645 l). However, no significant relationship was established between the polymorphism in intron 5 of the ABCG2 gene and milk yield across the different genotypes.

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ESTIMATION THE GENETIC PARAMETERS FOR MILK YIELD AND WOOL IN TURCANA BREED

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Abstract

The objective of this study was to estimate the breeding values and genetic parameters for milk yield in population of Țurcana breed and wool yield with animal model for selection. Data for milk yield consisted of records of 315 sheep and for wool yield records of 431 young sheep aged one year from Dacia County Association of sheep breeders. The mean for milk yield in milking period from sheep was 59±0.816 kg. The mean for wool yield for young sheep was 3.95±0.03kg. The studied population for milk yield of Turcana breed had two variety: Belă, 228 ewes with records and Breazǎ, 87 ewes with records. For wool yield the young sheep were 271 from Belă variety and 160 from Breaza variety. The breeding values for milk yield in milked period for sheep with records were between -3.56 and 5.55 and ranged between -5.65 and 5.55 in Țurcana population. The breeding values for wool production ranged between -0.412 and 0.631. The heritability for milk yield in milking period for Țurcana population was 0.21. The heritability for wool production was 0.34.

Key words*: animal model, genetic parameters, milk yield, sheep, wool production.*

INTRODUCTION

Țurcana is local sheep breed adapted to environmental condition of our country. All our native sheep breeds have mixed production, milk, meat and wool (Tafta, 2010).

The milk of sheep has biologique and economic importance, having a high level of protein and fat. Turcana is a rustic and resistant sheep breed, it is mixed morpho-productive type, being bred for milk, meat and wool. The mean of total milk yield during the lactation in Țurcana breed ranged between 90-120 kg (Pascal, 2015).

Țurcana is the most representative breed sheep in Romania (Pădeanu et al., 2015). Țurcana was reared in all the country. Țurcana is the oldest local breed in our country, being widespread in the submontane and mountain areas throughout the country and less in the plain areas (Taftă, 2010).

The factors which influence the milk production are genetic factors: breed, variety, individual. Age, prolificity, duration of lactation, level of nutrition, climate influenced the milk yield.

The varieties of Țurcana breed are: Țurcana Bucălaie, Țurcana Belă, Țurcana Breazǎ and Țurcana Oacheșă. Țurcana Belă is raised in counties: Alba, Bistrița, Cluj, Maramureș, Satu Mare, Gorj. Also, this variety is raised in Moldova. Țurcana Breaza is raised in Petrila, Lupeni, Straja in countie Hunedoara.

In the breeding program of Țurcana breed, the objective is the improvement of milk, meat and wool yields for the efficient and sustainable growth of the breed. Sheep breeding is done in an extensive system using a long period of the year the grazing and a short stable period the growth in shelters.

The milk yield of the Țurcana breed varies widely depending on variety, ecotype and welfare level. Țurcana Bucălaie produces 80-100 kg milk, Țurcana Belă 100-120 kg and Țurcana Breazǎ 120-150 kg. Țurcana Breaza has high milk yield during the lactation.

The total production of milk is given by the milk suckled by lambs and the milk milked. The period of standard lactation in Țurcana breed was 190 days, milking period was 120 days and suckling period was 70 days. For meat production, Țurcana young ewes obtained average daily gain over 190 g.

The wool production is influenced by breed, individual, age, sex, breeding, nutrition.

In Țurcana breeed the average wool production is 1.7-2.2 kg for ewes and 2.5-3.5 kg for rams. The white variety of Țurcana has a higher wool production with an average of 5 kg (Pascal, 2015).

The choice of the best method for genetic evaluation of ewes is very important for improvement the milk and wool production.
BLUP methodology applied to an animal model presents the following advantages: it uses information from all known relatives of an individual, it allows genetic comparisons betweeen animals that performed in different environments or different time periods, facilitates genetic comparisons between animals with different sources of information, allows genetic comparesons between animals that have been selected with different selection intensities, it makes possible the accurate measurement of the response to the selection (Grosu & Oltenacu, 2005).
The objective of this study was to estimate the breeding values and genetic parameters for milk yield in milking period and wool production in Țurcana with animal model for selection of the best sheep.

MATERIALS AND METHODS

The data used in this study provide from Dacia County Association of sheep breeders.
The pedigree contents 737 animals: 315 ewes with milk records, 117 sire and 305 dams from Țurcana breed. The ewes with records 315 from Țurcana breed are from 20 herds. The milk yield of ewes recorded in 2022, only first lactation was taken into account.
The pedigree for sheep with wool records consisted in 944 sheep: 431 young sheep with wool records, 99 sire and 414 dams. From 431 yearly ewes 381 were females and 50 were males. The sheep were from 25 herds. The wool production of young sheep was recorded in 2022, the quantity of wool from first cut.
The model used was animal model (Grosu & Oltenacu, 2005).
The model 1 can be written as:

yijk =Vi+Hj+ak+eijk

yijk = is the vector of observations, the phenotypic information was the total amount of milk after lambs suckling until the drying of the ewes;
Vi =is the fixed effect of variety of breed;
Hj= is the fixed herd effect;
a = is a sheep additive genetic effect;
e = is a residual error effect.
The model included the fixed effects: the variety of breed and the herd.

The model 2 can be written as:

yijk =Si+Hj+ak+eijk

yijk = is the vector of observations and is the quantity of wool from the first cut;
Si =is the fixed effect of sex;
Hj= is the fixed herd effect;
a = is a sheep additive genetic effect;
e = is a residual error effect.

The relative breeding value is:

$$BV\% = 100 + 6 * (\frac{BV_{abs} - Average\ BV_{abs}}{\sigma_{BVabs}})$$

BV% = relative breeding value;
BVabs = absolute breeding value;
σBVabs = standard deviation of absolute breeding values.
The breeding values and genetic parameters were calculated with R 4.1.2. software.
The values were checking with Grubs test if the values were in acceptable limits.

$$\hat{v} = \frac{X_{MIN,MAX} - \bar{X}}{\sigma}$$

$\hat{v} = the\ tested\ value$;
X= the value tested;
σ = standard deviation;
$\bar{X}$ = the mean.

RESULTS AND DISCUSSIONS

The breed has a great influence on the milk yield. Friza, Lacaune and Awassi are the breeds with high milk yield. The Țurcana breed has low milk yield compared to dairy sheep from Europe. The parity of sheep is a factor that influences the milk yield which it increases throughout the first three parities in sheep, in this study only record from the first lactation were considered, after which remained constant. The age of ewes influences the milk yield, up to 3-4 years the milk yield increases. The year of lactation and the lambing season influences the milk yield, the lambing in early spring extending the lactation period. The duration of lactation greater determines a high milk production. Food level influences the milk yield, a good nutrition determines high milk yield. Rational feeding of sheep based on category of age and category of production it is necessary in Țurcana breed. The main source of food was the green grass from pastures natural and cultivated and during the stable the food sources were represented by hay

and roughage, industrial residues, sillage fodder and combined fodder. During the lactation period the level of nutritional requirement increases (Pascal, 2015). Țurcana and Țigaia are the breeds in Romania with extensive system of growing based on transhumance. The high milk yield in Țurcana breed is in the second and third month of lactation. In the first 90 days of lactation obtained 65% from the total milk yield. In second part of lactation the milk yield decreases but increase the quality of milk by increasing the protein and fat levels.

The mean of milk yield in milking period for Țurcana breed was presented in Table 1.

Table 1. The descriptive statistics for milk yield in milking period in Țurcana breed for the first lactation

Specification	Țurcana	Țurcana Belă	Țurcana Brează
Mean± Standard error (kg)	59±0.816	58.95±0.8	59.14±2.04
Standard deviation	14.49	12.43	19.05
Coefficient of variability %	24.56	21.09	32.22
Number of ewes	315	228	87

In Table 2 are presented the means from BLUP that realized a reciprocal correction of effects in model. The standard errors associated with the results were computed from the square root of the diagonal of the generalized inverse (Mrode and Thompson, 2005).

Table 2. The means from BLUP solutions

Specification	Țurcana Belă	Țurcana Brează
Mean± Standard error (kg)	61.369±0.200	75.875±0.248

By Student test observed that were very significant difference between means from BLUP solutions for Țurcana Belă and Țurcana Brează for milk yield ($t_{calc} > t_{\infty;0.001}$) 6.6 >3.29. The milk yield in milking period was higher for the variety Țurcana Brează than for the variety Țurcana Belă, showing that the variety of Țurcana breed influenced the milk yield. In the population of Țurcana breed were ewes with milk yield in milking period higher than 100 kg milk, the maximum limit was 116.13 kg milk. All the ewes with milk yield higher than 100 kg milk were from variety Țurcana Brează. The second part of lactation takes place entirely during the grazing period.

In our study the milk yield in milking period was specific to the values of the breed. The coefficient of variability value was high showing a high variability in the population of variety Breaza. Kusza et al. (2018) reported the milk yield per lactation 76.8 kg in Țurcana breed. Trică et al. (2018) reported a mean of 76.81 kg milk in Țurcana breed. Sauer et al. (2016) reported milk yield in Țurcana ewes on average 77.98 kg.

In the suckling period an average milk yield of 92.23 during the first 85 days of lactation was reported by Pascal et al. (2010) in Țurcana breed. Taftă (2010) showed that the local sheep breeds have a great variability in milk, meat and wool. Puie et al. (2020) reported a mean of 82 kg milk in Bălă ecotype of the Țurcana breed. Caraba (2023) showed that an efficient feeding system for sheep determines an increase in the quantity and quality of milk in Țurcana breed.

The mean of wool production is presented in Table 3.

Table 3. The descriptive statistics for wool production in Țurcana breed for the first cut

Specification	Țurcana	Males	Females
Mean±Standard error (kg)	3.95±0.03	5.37±0.1	3.76±0.023
Standard deviation	0.73	0.85	0.45
Coefficient of variability %	18.51	15.82	12.17
Number of yearling ewes	431	50	381

For the selection of ewes for milk yield it is necessary the selection for the size of mammary gland, duration of lactation, the productive longevity, the prolificity, the weight of lambs at 20 days.

Table 4. The means for sex effects from BLUP solutions

Specification	Males	Females
Mean±Standard error (kg)	5.421±0.023	3.637±0.012

In Table 4 we can observe the BLUP means separated for the two sexes.

By Student test observed that were very significant difference between means from

BLUP solutions for males and females for wool ($t\ calc > t_{\infty;0.001}$) 14.55>3.29.

The wool production was higher for males 5.37 kg than for females 3.76 kg, showing that the sex had a great influence of the wool yield. The rams of both variety Belă and Breazǎ had a high wool production over 5 kg. The white variety of Țurcana is appreciate for long white wool. The yield of wool at washing was 65-70%.

Pădeanu et al. (2015) reported a mean of 3.94 kg in Țurcana yearling ewes. To increase wool production and improve quality, the selection methods must be combined with those of feeding ensuring a higher protein level. The selection of sheep for wool production is based on quantity, fineness, homogeneity, length, thickness, washing yield (Taftă, 2010).

The factor that influences the wool production is the breed. Mortimer et al. (2017) reported the yearling greasy fleece weight in Merino breed was 3.97 kg and the yearling clean fleece weight was 2.79 kg. The age of sheep influences the wool yield was higher at adult sheep. The higher quantity of wool was obtained until 5-6 years. Taftă et al. (2010) reported that the wool production depens the following factors: breed, individual, age, sex, level of selection, nutrition, and harvesting technique. The higher productions are in the Merino breeds and lower yield in the breeds with thick wool.

The breeding values for the milk yield in milking period for the best ewes with records in Țurcana breed sheep were presented in the Table 5.

Table 5. The breeding values for the 10 best sheep for milk yield

No	Absolute breeding values	Relative breeding values
1	3.979	121.20
2	3.815	120.37
3	3.735	119.97
4	3.629	119.44
5	3.433	118.45
6	2.998	116.26
7	2.989	116.21
8	2.666	114.59
9	2.666	114.59
10	2.644	114.48

The absolute breeding values ranged between 2.644 and 3.979 for the best ewes. The relative breeding values for the best ewes ranged between 114.48 and 121.20. The individuality within in Țurcana breed is represented by a significant proportion of plus variants which under the same feeeding and environmental conditions produce a higher milk yield than the herd average. The higher breeding values for milk yield indicates the sheep's genetic merit. The breeding values are important for include in the calculation of the selection index values for rank the animals. The farmers will use rams with a higher breeding value for make fast, reliable and more predictable genetic gain in milk production traits. The breeding values for milk yield in milked period for sheep with records were between -3.485 and 3.979 and ranged between -3.56 and 3.979 in Turcana population. The wide range of estimated breeding values can be attributed to the high genetic variation in the Țurcana breed and variety. Jawasreh et al. (2022) reported the breeding values ranged between -36.34 to 72.01 in Awassi sheep. Jawasreh and Khasawneh (2007) obtained the breeding values between -205.1 and 441.2 kg in Awassi breed. Rotar et al. (2017) realized a comparison of breeding value methods for milk production in flock of Palas milk line and constated that the best results were obtained with the combination of the Individual Animal Model and Lush index. Popa et al. (2020) obtained the breeding values for the best Teleorman Black Head sheep between 14.56 and 28.48 kg.

In the Table 6 were presented the breeding values for the best yearling sheep for wool production.

Table 6. The breeding values for the 10 best sheep for wool production

No	Absolute breeding values	Relative breeding values
1	0.416	127.73
2	0.381	125.39
3	0.349	123.26
4	0.323	121.52
5	0.32	121.32
6	0.305	120.32
7	0.294	119.58
8	0.269	117.92
9	0.26	117.31
10	0.252	116.78

The absolute breeding values for the best yearling ewes for wool yield ranged between 0.252 and 0.416. The relative breeding values for the best ewes ranged between 116.78 and 127.73. The breeding values for wool production

ranged between -0.369 and 0.416. The estimated breeding values ranged wide showing a high genetic variation in studied population. In the population of Țurcana were sheep with high performances than the herd average for wool yield. Daetwyler et al. (2010) reported genomic estimated breeding values for wool traits in Merino sheep ranged from 0.15 to 0.79. The breeding values of sheep depends of the variability from breeds. Moghaddar et al. (2014) reported the accuracy of genomic estimated breeding values of yearling and adult wool traits in Merinos was an average high (0.33-0.75).
In the Table 7 was shown the heritability for milk yield in milked period and wool production in two populations of Țurcana breed.

Table 7. The heritability for milk yield and wool production in the populations of Țurcana breed

Item	Milk yield	Wool production
h^2	0.21	0.34

The heritability for milk yield in Țurcana breed was 0.21 and the heritability for wool yield was 0.34. Barillet et al. (2001) reported the heritability for milk yield in Lacaune sheep 0.34. El-Saied et al. (1999) found a heritability of 0.24 for milk yield in Spanish Churra and Ligda et al. (2002) reported a heritability of 0.35 in Greek Chios sheep breed. The heritability for wool yield in our study was lower than heritability reported by Singh et al. (2019), Hanford et al. (2002) 0.53 for fleece weight in Columbia sheep, Bromley et al. (2000) 0.47 and Fogarty et al. (1995) 0.36. Mortimer et al. (2017) reported the heritability for yearling gresy fleece 0.57 and for yearling clean fleece 0.52. Safari et al. (2005) reported mean heritabilities for greasy fleece weight in wool breeds 0.37 and dual-purpose breeds 0.38 and for clean fleece weight in wool breeds 0.36. By genetic improvement of the breed it is possible to increase sheep milk and wool yields efficiently and sustainably (Flint and Woolliams, 2008). Implementation selection programs in smaller flock can has positive economic and social impacts, preserve breeds and facilitate the genetic progress in sheep populations (Marshall et al., 2024).

CONCLUSIONS

The selection of sheep for milk yield and wool yield must realize take in consideration the breeding values. The heritability for milk yield was low 0.21 and for wool production was moderate 0.34 ranged in the values reported in literature.

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IMPACT OF STIMULATION DIETS DURING PRE- AND POSTPARTUM PERIODS ON SHEEP LACTATION

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Abstract

The prepartum period (last 4-6 weeks of gestation) and the postpartum period (first 6-8 weeks after calving) are critical for the success of lactation. Sheep require adequate nutrition for optimal lamb development, preparation of the mammary glands for lactation, avoidance of metabolic problems (ketosis, hypocalcemia). A stimulation diet must provide: sufficient energy (through quality hay, organic cereals), good quality proteins (alfalfa, forage legumes, organic soybeans), essential vitamins and minerals (vitamin E, selenium, calcium, phosphorus). The aim of this study was to monitor the effect of administering a feed complex prepared in an organic dairy sheep farm and a supplement of organic concentrates and vitamin-mineral premix to determine the increase in production and quality of milk produced. Organically raised ewes fed with peripartum and postpartum stimulation diets achieved significantly higher milk production compared to ewes not receiving supplementary feed, the differences being highly statistically significant. The values of the physicochemical parameters analyzed in milk from ewes with supplementary feed indicated an improvement in milk quality, with an optimal milk composition (fat, protein, lactose, casein).

Key words*: concentrate supplements, dairy sheep, milk production, nutrition.*

INTRODUCTION

The productivity of farm animals (sheep, goats) is determined by their genotype and is influenced by their health status and the environment in which they are raised. By the term "rearing environment" we understand the housing conditions, the health of the animals and their nutrition (Nguyen, 2022). Nutrition is a fundamental element in the raising of all production animals, as it directly influences their performance and health (Bencini et al., 2010). It should be noted, however, that the topic of sheep nutrition, especially dairy ones, is quite complex and a nutrition plan with general applicability in all farms cannot be created. Thus, sheep nutrition must adapt to the recommendations of specialists in the field in order to optimally cover the nutritional needs of dairy sheep raised on farms with different breeding systems.

Stimulating milk production in organic farms with early lambing sheep in winter is a complex process that involves both nutritional strategies and management measures adapted to the specific winter conditions in terms of diet, mineral and vitamin supplementation, and stress management. During this period, ewes have to cope with a double stress: adapting to cold conditions and stimulating lactation, which can be more difficult due to higher energy and protein requirements.

Sheep with a balanced diet during this period have an easier calving, produce higher quality colostrum, start lactation in an optimal physiological state. This way of feeding allows for vigorous lambs at birth, a satisfactory production of colostrum, which allows the lambs to receive the antibodies necessary for their passive immunity, thus reducing the perinatal mortality rate and allowing a good stimulation of milk production, which will be increased both in quantity produced and in the duration of lactation (Tufarelli et al., 2009). After lambing, the main objective is to maintain milk production and quickly recover the sheep for the next reproductive cycle. A

well-formulated diet, rich in protein, energy, vitamins and minerals, together with comfortable housing and constant access to water, will help ensure efficient milk production and the general health of the sheep and lambs (Pulina et al., 2006). The standards that regulate feed management in organic systems represent one of the most critical factors influencing milk production performance (Hernandez et al., 2016). Adapting the diet and management to meet the specific requirements of this period will contribute significantly to the success of an organic dairy sheep farming system. The impact of pre- and postpartum stimulation diets on lactation in organically raised sheep is a topic of great interest in animal husbandry, with direct implications for milk production, animal health and the sustainability of organic farming systems.

As a result, chemical analyses of the available feed (fibrous and concentrate) and the rations administered should be carried out at regular intervals. The last point is particularly important, because in practice it is proven that, in the steps that intervene between the development of a ration, for example, with the help of a computer, the mixing of the feed and its provision to the animals, the initial ration changes. These changes are due either to the lack of infrastructure for precise weighing of the ration components, or to the inattention of the staff involved in animal feeding.

Factors that influence feed intake are: the form, type and method of ration administration, the quality of fibrous feed, the ratio of concentrates/fibrous feed in the ration, the protein content of the ration, the size of the rumen, previous restrictions, the age of the animals, the physiological stage they are in, the availability, quality and consumption of water (Zervas & Tsiplakou, 2011).

The nutritional needs of dairy sheep vary significantly depending on the lactation period, the age of the animal and the physiological state (e.g. pregnancy, lactation). In the second part of gestation, especially in the last month of gestation and in the first part of lactation, the need for protein, energy and minerals is much higher (Francois & Caja, 2004). During this period, the reduced feed intake capacity, combined with the high energy requirements for fetal growth (in the prepartum period) and milk production (in the postpartum period), represents a significant metabolic challenge for lactating ruminants, therefore, during this period the need for energy will increase by 20-30% and for protein by 30-40% (Zarrin et al., 2021). Special attention must be paid to ensuring mineral substances (calcium, phosphorus) and vitamins A, D and E (Selmi et al., 2019).

The watering is an essential element for milk production. It is important that sheep have constant access to clean and sufficient water, since milk is mostly water and proper hydration contributes to optimal lactation.

The proper management of animal nutrition, with the selection and provision of appropriate feeds, is essential for achieving high performance and maintaining their health (Morand-Fehr et al., 2007).

This study aimed to analyze the influence of two variants of concentrated feed supplements administered during the prepartum (last 4 weeks of gestation) and postpartum (90 days after calving) periods on milk production obtained from crossbred ewes raised in an organic system.

MATERIALS AND METHODS

The organization of the experiment was conducted in accordance with the provisions of Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, as well as with all applicable national legislation governing animal welfare under experimental conditions. All procedures were reviewed and approved by the institutional bioethics committee.

The milk analyzed came from crossbred Lacaune and Karagouniko ewes raised organically on a farm located in the central region of Greece (Latitude: ~39.32° N; Longitude: ~22.52° E). The milk was collected over a 3-month period (January, February, March, 2025), during which the ewes were physiologically in the postpartum period.

The nutrient requirements of animals depend on a series of factors (species, sex, breed, age, body weight, quantitative and qualitative level of specific production), which condition the extent of the requirements, the degree of feed utilization in the elaborated production, as premises for establishing the feeding norms

corresponding to the different physiological states (rest, mating, gestation, lactation) and productive levels (Sanz Sampelayo et al., 2007).

Stimulating milk production in organically raised ewes in the event of early lambing is essential for the success of a sustainable and efficient farming system. Early lambing, which occurs at the beginning of the winter season or even before, can put considerable pressure on the animals, especially in terms of energy and protein requirements for milk production and lamb development.

From Figure 1 it can be seen that the most critical periods, in terms of meeting the needs of ewes, are autumn (the last stage of pregnancy) and winter (the first phase of lactation - suckling the lambs), when there is no pasture available.

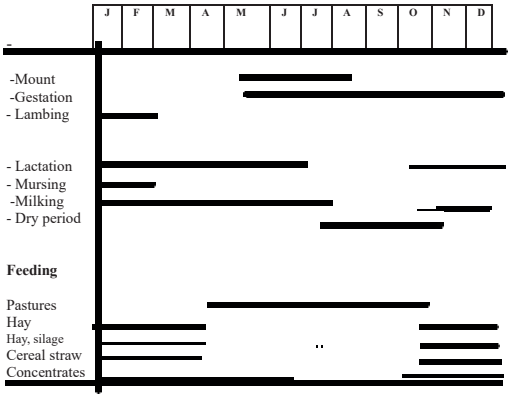


Figure 1. Schematic presentation of sheep rearing technology

During these periods, the farmer must provide the animals with sufficient and balanced supplementary feed, so that their physical condition does not have negative effects on milk production and, subsequently, on their fertility.

In the case of early lambing, the ewes are in a period of stress, as they have to adapt to the demands of lactation, even in the more difficult conditions of cold climate and more restricted feeding, since during winter, pastures are limited and the available feed may not be sufficient to meet the high energy and protein requirements necessary for lactation. The ewes may have less energy available to support milk production if they do not receive an adequate supply of nutrients.

In order to assess the effectiveness of lactation stimulation strategies, it is important to continuously monitor the health of the ewes and milk production. This may include: observing the amount of milk produced daily, analyzing the quality of the milk produced, checking the health of the mammary gland to prevent mastitis and other infections, as well as assessing the general condition of the animal, including body weight and animal behavior. (Nudda et al., 2014)

In this study, 120 crossbred ewes aged between 2 and 3 years were selected from a herd of 550 ewes raised organically in a semi-intensive farming system, which calved in the same month. The ewes were divided into two experimental groups and were fed according to the experimental scheme presented in Table 1.

The sheep were selected from the entire farm, before the start of the experimental period, being in the same physiological state of advanced gestation and were divided into two experimental groups (A and B), homogeneous in terms of physiology and age (2-3 years):

- Group A consisted of 60 sheep that were fed with feed from their own production, namely bulk feed (75% of the ration): 1.0 kg of clover silage, 1.0 kg of hay silage composed of legumes and grasses (peas, peas, wheat, oats, barley), 0.5 kg of clover hay, 0.5 kg of wheat and barley straw and organic concentrated feed (25% of the ration), respectively 1.0 kg of a mixture of organic cereals and legumes (0.6 kg of corn, 0.2 kg of barley, 0.2 kg of unmodified soybeans genetically), administered throughout the day, for one month before calving and until the end of the lactation period.

- Group B consisted of 60 sheep that received the same feed as group A, produced within the farm, in the same quantity. In addition, the sheep's ration was supplemented with 0.6 kg of combined feed to stimulate milk production with the addition of a premix containing essential vitamins, macroelements and mineral trace elements. This combined feed was administered in an amount of 0.6 kg/head/day in the first 45 days after calving and 0.3 kg/head/day in the milking period for another 45 days, in two feedings per day.

No food selectivity was observed, and no hay or concentrate residues were left after each feeding. Water was provided *ad libitum*.

Table 1. Experimental scheme

Experimental group	n	Administered feeds		Analyzed parameters
		Basic ration	Concentrate mix	
Group A	60	Roughage (75% of the ration): - 0.5 kg/head/day clover hay - 0.5 kg/head/day wheat and barley straw - 1.0 kg/head/day clover silage -1.0 kg/head/day hay silage composed of legumes and grasses (peas, beans, wheat, oats, barley)	Organic concentrate feed (25% of the ration): - 1.0 kg/head/day mixture of organic cereals and legumes (0.5 kg corn, 0.2 kg barley, 0.2 kg non-genetically modified soybeans), 0.1 kg vitamin-mineral premix; - administration for one month before calving and until the end of the experimental period (90 days after calving)	- milk production; - physicochemical and microbiological parameters of milk.
Group B	60	Roughage (65% of the ration): - 0.5 kg clover hay - 0.5 kg wheat and barley straw - 1.0 kg clover silage - 1.0 kg hay silage composed of legumes and grasses (peas, beans, wheat, oats, barley)	Organic concentrates (35% of the ration): - 1.0 kg/head/day mixture of organic cereals and legumes similar to Group A - 0.6 kg/head/day combined feed to stimulate milk production administered for one month before calving and during the suckling period (45 days-period I) and 0.3 kg/head/day in milking period (45 days-period II)	

The preparation of the total mixed ration on the farm involves a process of precise measurement, adequate preparation of the ingredients, uniform mixing and continuous monitoring using appropriate equipment (grain mill, chopper, mixer) with which all the ingredients will be combined into a homogeneous ration, without the hay remaining isolated or the silage being divided unevenly. The hay and silage were cut into smaller pieces to improve the mixture and prevent selection by the animals, using a chopper at a width of approximately 3-5 cm, to be more easily digestible. The cereals were ground using a grain mill, so that they were more easily digestible by the sheep. After measuring each ingredient, their combination was carried out using the mixer and finally, uniform distribution in the animal shelter using the distributor. If the mixture is not homogeneous, there is a risk that some animals receive a diet richer in some ingredients and poorer in others, which could lead to nutritional imbalances.

The compound feed administered with the role of stimulating milk production (Tables 2 and 3) was analyzed to determine the chemical composition, according to Commission Implementing Regulation (EU) 2024/771 on the methods of sampling and analysis for the official control of animal feed.

The sheep were milked twice a day, and the amount of milk was recorded separately for each sheep. The daily milk production of each sheep was recorded using graduated cylinders attached to individual milking units, during the experimental period, for two consecutive days, at the beginning of weeks 1, 2, 3, 4, 5, 6 of lactation (the period of lambing) and at the beginning of weeks 7, 8, 9, 10, 11, 12 of lactation (after lambing).

Table 2. Composition of the compound feed administered to sheep from group B

No.	Ingredients	Amount (%)
1	Maize	38.00
2	Barley	10.00
3	Non-genetically modified soybean meal	10.00
4	Maize DDGS (Dry Distillers Grains with solubles)	10.00
5	Wheat bran	12.00
6	Sunflower meal	11.80
7	Vegetable oil	1.50
8	Molasses	2.50
9	Calcium carbonate	1.40
10	Calcium diphosphate	0.40
11	Monosodium sulfate	0.50
12	Salt	0.50
13	Vitamin-mineral premix*	1.40
	Total	100.00

*The vitamin-mineral premix (MP-28 Power Mpletsas, Ioannina, Greece) contains the following analytical components, reported per 1 kg of product, according to the technical sheet: vitamin A 15,000 IU, vitamin D3 3,000 IU, vitamin E 300 mg, vitamin K32 mg, vitamin B15 mg, vitamin B2 3 mg, vitamin B6 0.2 mg, vitamin B12 30 mg, vitamin C 50 mg, biotin 2.5 mg, pantothenic acid 10 mg, inactivated yeast $30*10^9$, nicotinic acid 20 mg, folic acid 0.25 mg, choline chloride 200 mg, iron 50 mg, zinc oxide 70 mg, organic zinc 50 mg, zinc sulfate 90 mg, manganese oxide 45 mg, iodine 3 mg, cobalt 0.4 mg, sodium selenite 0.35 mg, organic selenium 0.24 mg.

Table 3. Chemical composition of the compound feed administered to sheep from group B

Chemical composition	Amount (%)	Analysis method
Moisture	11.64	Reg.(EU)771/2024
Dry matter	88.36	Calculated
Crude protein	19.05	Reg.(EU)771/2024
Crude cellulose	5.65	Reg.(EU)771/2024
Crude fat	5.33	Reg.(EU)771/2024
Crude ash	6.47	Reg.(EU)771/2024
Calcium	0.83	AOAC 968.08:2006
Phosphorus	0.64	AOAC 968.08:2006
Magnesium	0.35	AOAC 968.08:2006
Sodium	0.35	AOAC 968.08:2006

Milk samples for physicochemical and microbiological analyses were taken in sterile containers, respecting hygiene standards to avoid secondary contamination at the beginning of each week, until the end of the experimental period. Milk from ewes in the same group was collected separately.

After homogenization, three partial samples were taken from the total quantity of milk from each batch, which were homogenized, and the resulting sample constituted the laboratory sample, on which the following analyses were performed: physical parameters (pH determined immediately on the farm, density), chemical parameters (lactose, fat, protein, casein, total dry matter, non-fat dry matter), as well as microbiological analyses.

The milk was stored and transported at a temperature of +4°C, and the analyses were performed in the laboratory within approximately two hours of sampling.

The pH value was measured using a portable multimeter Multi 340i/SET WTW (Weilheim, Germany), equipped with a specific sensor for each parameter. The pH determination was performed immediately after milking, and calibration was performed with standard buffer solutions at pH 4.0 and 7.0, according to the manufacturer's instructions.

The chemical composition of the milk was determined by infrared stereoscopy, using a MilkoScan FT3, Foss Analytical A/S, (Hillerød, Denmark).

The determination of microbiological parameters was performed using the BactoScan™ FC+ bacteria analyzer, Foss Analytical A/S, (Hillerød, Denmark), equipment used for the rapid and accurate determination of the total number of bacteria in milk, which operates on the principle of flow cytometry, providing results in CFU/ml (colony forming units per milliliter).

For the statistical analysis of the experimental data, Microsoft Excel was used to calculate the mean, standard error of the mean, standard deviation (s). The Student t test was used to compare the means of two groups and check whether the difference between them is statistically significant. The program used displays a value called p-value, which can be $p>0.05$ (NS - the difference between the means is not significant), $p<0.05$ (* - the difference between the means is significant), $p<0.01$ (** - the difference is distinctly significant), $p<0.001$ (*** - the difference is very significant).

RESULTS AND DISCUSSIONS

Table 4 shows the evolution of milk production during the lactation period of lambs over six consecutive weeks in two experimental groups, Group A and Group B, each consisting of 60 ewes.

Throughout all weeks, Group B showed a constant and significantly higher milk production compared to Group A. In Week 1, the average milk production in Group B was 1128 ± 6.94 ml/head/day, exceeding Group A, which recorded 955 ± 8.60 ml/head/day ($p < 0.001$). This highly significant difference was maintained throughout the study period. By Week 6, Group B reached 1339 ± 7.23 ml/head/day, while Group A recorded 1061 ± 7.04 ml/head/day, reflecting an overall superior lactation performance in Group B.

The fluctuations in milk production over the weeks reveal a general upward trend from Week 1 to Week 3, with a slight decrease in Week 4, followed by stable or slightly increased values in the following weeks. Group A peaked in Week 3 (1083 ± 7.91 ml/head/day) and then showed a marginal decrease, while Group B showed a more sustained increase, peaking in Week 6 (1339 ± 7.23 ml/head/day).

The average daily milk production demonstrates the effect of administering the combined feed supplement to sheep in Group B, which recorded a higher productivity (1228 ± 7.35 ml/head/day) compared to sheep in Group A (1019 ± 7.74 ml/head/day), the difference being highly statistically significant ($p < 0.001$). In addition, the standard deviations (s) in both groups indicate moderate variability within the groups, although Group B consistently maintained a slightly lower variability, suggesting a more homogeneous performance.

Table 5 illustrates the progression of milk production during a 45-day milking period of the sheep in the two experimental groups, Group A and Group B, each with a number of 60 sheep.

Throughout the entire experimental period, the sheep in Group B demonstrated a constant and significantly higher milk production compared

to Group A ($p < 0.001$ for all weeks). In Week 1, Group B recorded an average milk production of 2362 ± 19.98 ml/head/day, clearly exceeding Group A, which recorded 1925 ± 19.68 ml/head/day. This situation was manifested during all subsequent weeks, with Group B maintaining a substantial advantage in terms of production. The peak milk production in Group B occurred in Week 5 (2417 ± 17.12 ml/head/day), while ewes in Group A showed minimal variation from week to week, indicating a plateau in production capacity.

The calculated average daily milk production underlines these observations, with ewes in Group B reaching 2389 ± 17.21 ml/head/day, significantly higher than that of Group A, 1924 ± 18.23 ml/head/day ($p < 0.001$). The standard deviations (s) in both groups indicate moderate within-group variability, which remained comparable throughout the study period. It is important to note that despite the higher absolute production levels, Group B maintained a similar variability to Group A, suggesting that the increased yield was exhibited by all ewes.

Table 6 presents the evolution of the different physicochemical parameters of the milk obtained from the two experimental groups (Group A and Group B) during two distinct periods, suckling period (period I) and milking period (period II).

Table 4. Evolution of milk production obtained during suckling period (45 days) (ml/head/day)

Specification	Group A			Group B		
	n	M±SEM	s	n	M±SEM	s
Week 1	60	955±8.60	93.86	60	1128±6.94***	75.72
Week 2	60	946±7.52	82.08	60	1132±7.55***	82.36
Week 3	60	1083±7.91	86.29	60	1289±8.21***	89.54
Week 4	60	1050±7.60	82.95	60	1214±6.52***	71.08
Week 5	60	1020±7.77	84.72	60	1264±7.65***	83.47
Week 6	60	1061±7.04	76.83	60	1339±7.23***	78.87
Average daily milk production		1019±7.74	84.45		1228±7.35***	80.17

M = mean; SEM = standard error of the mean; s = standard deviation

Table 5. Evolution of milk production obtained during milking period (45 days) (ml/head/day)

Specification	Group A			Group B		
	n	M±SEM	s	n	M±SEM	s
Week 1	60	1925±19.68	214.73	60	2362±19.98***	218.01
Week 2	60	1922±19.01	207.42	60	2367±18.21***	198.60
Week 3	60	1918±18.57	202.62	60	2394±16.65***	181.67
Week 4	60	1921±17.88	195.09	60	2396±16.74***	182.59
Week 5	60	1927±17.37	189.46	60	2417±17.12***	186.85
Week 6	60	1931±16.86	183.92	60	2398±17.48***	190.70
Average daily milk production		1924±18.23	198.87		2389±17.21***	193.07

Table 6. Physico-chemical parameters of milk analyzed in the experimental period

Parameters	Group A		Group B		
	M±SEM	s	M±SEM	s	p
Density (g/l)					
Period I	1.986±0.057	0.128	2.370±0.053***	0.118	0.00061
Period II	1.035±0.0003	0.0007	$1.035±0.0002^{NS}$	0.0006	0.687
pH					
Period I	6.82±0.015	0.034	$6.81±0.015^{NS}$	0.033	1.00
Period II	6.80±0.005	0.011	$6.81±0.004^{NS}$	0.009	0.450
Total dry matter (%)					
Period I	16.73±0.131	0.289	16.84±0.080*	0.174	0.491
Period II	16.40±0.111	0.249	17.88±0.065***	0.146	0.00003
Non-fat dry matter (%)					
Period I	11.27±0.046	0.102	10.72±0.093***	0.208	0.00035
Period II	10.24±0.053	0.119	11.56±0.040***	0.090	0.0000006
Crude fat (%)					
Period I	5.92±0.097	0.216	6.23±0.079*	0.176	0.031
Period II	5.92±0.063	0.141	6.73±0.033***	0.074	0.0000005
Crude protein (%)					

Parameters	Group A		Group B		
	M±SEM	s		M±SEM	s
Period I	5.35±0.054	0.121	5.34±0.020^{NS}	0.045	0.822
Period II	5.28±0.045	0.102	5.56±0.087*	0.195	0.017
Casein (%)					
Period I	4.08±0.030	0.067	4.08±0.053^{NS}	0.119	1
Period II	4.02±0.038	0.084	4.16±0.023**	0.051	0.009
Lactose (%)					
Period I	4.75±0.031	0.069	4.75±0.063^{NS}	0.141	1
Period II	4.48±0.028	0.062	4.77±0.081**	0.182	0.007
Glucose (%)					
Period I	0.17±0.010	0.023	0.16±0.007^{NS}	0.017	0.446
Period II	0.20±0.007	0.016	0.22±0.007^{NS}	0.015	0.153
Galactose (%)					
Period I	0.10±0.016	0.036	0.08±0.013^{NS}	0.030	0.363
Period II	0.16±0.009	0.022	0.16±0.009^{NS}	0.021	1
Total Microbial Flora (cfu/ml)					
Period I	63,000±3,781	6,455	58,000±1,468^{NS}	3,283	0.068
Period II	80,166±5,728	5,863	51,000±1,913^{NS}	4,459	0.062

In Period I, the milk density was significantly higher in Group B (2.370 ± 0.053 g/l) compared to Group A (1.986 ± 0.057 g/l, $p<0.001$), indicating a notable improvement in the milk composition as a result of the administration of the dietary supplement. However, no significant difference was observed between the groups in Period II ($p = 0.687$).

The milk pH remained similar between the groups during both periods, with no statistically significant differences ($p > 0.05$), suggesting that dietary supplementation did not influence the acidity or alkalinity of the milk.

Although a minor, non-significant increase was observed in Group B during Period I (16.84 ± 0.080%) compared to Group A (16.73 ± 0.131%, $p = 0.491$), a highly significant increase occurred in Period II (17.88 ± 0.065% vs. 16.40 ± 0.111%, $p = 0.00003$). This indicates an improved nutritional density of milk over time due to the supplementary diet. Group B showed a significantly lower SNG content in Period I (10.72 ± 0.093%) compared to Group A (11.27 ± 0.046%, $p = 0.00035$).

However, in Period II, the trend was reversed, with Group B showing a significantly higher level of SNF (11.56 ± 0.040%) than Group A (10.24 ± 0.053%, $p = 0.0000006$). This suggests a positive impact of the diet over time.

Fat content was moderately but significantly higher in Group B in both periods. In Period I, Group B recorded 6.23 ± 0.079% versus 5.92 ± 0.097% in Group A ($p = 0.031$). In Period II, the difference became highly significant (6.73 ± 0.033% versus 5.92 ± 0.063%, $p = 0.0000005$), confirming the strong effect of supplementation on fat synthesis.

Protein content was similar between groups in Period I ($p = 0.822$). However, by Period II, Group B demonstrated a significantly higher protein level (5.56 ± 0.087%) compared to Group A (5.28 ± 0.045%, $p = 0.017$), supporting the hypothesis that dietary supplementation improved milk protein synthesis over time.

Casein content was comparable in Period I ($p = 1.00$). A significant increase in casein was observed in Group B in Period II (4.16 ± 0.023% vs. 4.02 ± 0.038%, $p = 0.009$), which is important for improving cheesemaking potential and nutritional value of milk.

No differences in lactose content were detected in Period I ($p = 1.00$). In Period II, however, Group B showed a distinctly significantly higher lactose content (4.77 ± 0.081%) compared to Group A (4.48 ± 0.028%, $p = 0.007$), indicating an increased carbohydrate content.

No statistically significant differences were observed in either glucose or galactose concentrations between the two groups in either period (all $p > 0.15$), suggesting that these carbohydrate fractions were not affected by the dietary treatment.

Although not statistically significant, Group B consistently showed lower numbers of microorganisms in both periods.

From the analysis of the results obtained, it can be appreciated that the ewes in Group B, which received a supplement of combined feeds both in the prepartum and postpartum periods,

showed significantly higher milk yields compared to the ewes in Group A, both in the lactation and milking periods.

The results showed that ewes in Group B, which received a dietary supplement both prepartum and postpartum, had significantly higher milk yields during both lactation and milking. This is in line with the findings of Zarrin et al. (2021), who reported that pre- and postpartum dietary restrictions negatively affected colostrum and milk yields in fat-tailed dairy ewes, highlighting the importance of adequate nutrition during these periods.

Similarly, studies have shown that pre- and postpartum supplementation with energy-rich feeds such as cracked corn can double colostrum production in ewes, thereby enhancing neonatal nutrition and immunity (Banchero et al., 2004).

Milk from ewes in Group B also had higher total solids, fat, protein and lactose content, especially during the postpartum period. This is in line with the study by de Sousa et al. (2018), who found that dietary supplementation in Santa Inês sheep during the pre- and postpartum periods improved milk production and influenced mineral metabolism.

Regarding microbial quality, although our study observed a lower number of microbes in Group B, the differences were not statistically significant. Maintaining a low somatic cell count is essential for milk quality, and nutritional strategies during the transition period can influence this parameter (Morand-Fehr et al., 2007).

The results obtained highlight the positive effects of specific nutritional supplementation during the pre- and postpartum periods on milk production and quality in sheep. These results are consistent with the existing literature, which highlights the significant role of nutritional and management practices in optimizing milk production and composition in sheep.

CONCLUSIONS

Administration of a concentrate feed supplement to sheep in Group B positively influenced milk production throughout the suckling period, leading to higher and more stable yields compared to Group A.

The same situation is observed in the case of the lactation period, the dietary stimulant administered to sheep in group B having a substantial and sustained positive effect on milk production during the milking period, leading to higher and more stable yields compared to Group A.

The combined feed supplement administered to Group B had a clear and significant positive impact on several key physico-chemical properties of milk, particularly in Period II. Enhancements in total solids, fat, protein, casein, and lactose content, along with trends toward lower microbial counts, indicate improved milk quality and nutritional value associated with the dietary intervention.

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ESTIMATES OF METHANE ENTERIC EMISSIONS FROM THE ROMANIAN DAIRY CATTLE SECTOR BETWEEN 2015-2024

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Abstract

The aim of this paper was to estimate methane emissions resulted from the enteric fermentation of dairy cattle, between 2015 and 2024 in Romania. The number of dairy cattle used in this paper was reported by the National Institute of Statistics, with enteric emissions being estimated using Tier 1 and Tier 2 methodologies provided by the International Panel on Climate Change Guidelines (IPCC, 2006) and expressed as Gg CH_4 yr^{-1} and t CO_2-eq. The gross energy intake (GE), digestible energy (DE), and methane conversion factor (Y_m) values for Tier 2, were calculated according to national reference values. Total methane emissions estimated for dairy cattle, decreased from 2.972.264 t CO_2-eq/year to 2.672.010 t CO_2-eq/year for Tier 1 and from 2.745.591 t CO_2-eq/year to 2.468.235 t CO_2-eq/year for Tier 2, from 2015 to 2024. The decrease in methane emissions was attributed to the decrease of dairy cattle numbers. The results can provide information on the status quo of the dairy industry, when the environmental footprint is concerned, as well as benchmark information in order to develop appropriate future strategies to reduce carbon footprint of the cattle sector.

Key words*: dairy cattle, enteric fermentation, methane emissions.*

INTRODUCTION

The livestock sector represents an anthropogenic source of greenhouse gases (GHG), and thus contributes to climate change (Vagoni et al., 2019).

Given their digestive system, ruminants are responsible for the increasing CH_4 emissions at the global level (Ghassemi-Nejad et al., 2024), with methane resulting from enteric fermentation, representing approximately 35-70% of the carbon footprint (CF) of milk produced (Mc Geough et al., 2012).

Historical studies have shown that global enteric CH_4 emissions started to increase from the middle of the 19th century (Garnier et al., 2019), with the intensification of the dairy industry and the increase of livestock units.

The production of methane varies significantly according to the nature of the feed and the fermentation intensity (accounting for 15-30% of the total ruminal gas), and it is positively correlated with the animal live weight and diet characteristics, this method is recommended for the development of country-specific factors to enhance the accuracy of enteric CH_4 estimation. The Tier 3 method is applying to a national inventory when comprehensive production levels (Popa et al., 2017; Tubiello et al., 2013).

To calculate CH_4 emissions from ruminants, a set of equations provided by the International Panel on Climate Change Guidelines (IPCC, 2006) was developed.

The IPCC suggested a three tier (Tier 1, Tier 2, and Tier 3) approach for national GHG inventories (Jo et al., 2016), with the recommended development of region-specific emission factors (RSEF). The Tier 1 method involves default values for emission factors, and the data are based on previous studies, presenting the emission factors by region based on animal numbers.

The Tier 2 method is an improved and more complex method that requires information about animal categories, feeding and production systems, and manure management. The calculation is based on the gross energy (GE) intake and conversion rate of gross energy intake into methane energy (Y_m), which can be chosen according to the level of productivity and knowledge of all factors influencing enteric and manure CH_4 emissions can be considered. This method is complex and laborious, requiring a series of exact region – specific factors.

The aim of this paper was to estimate methane (CH_4) emissions resulted from the enteric fermentation of dairy cattle, between 2015 and 2024 in Romania.

MATERIALS AND METHODS

The number of dairy cattle used in this paper to estimate CH_4 emissions was reported by the National Institute of Statistics (INS, 2024).
The enteric CH_4 emissions were estimated using Tier 1 and Tier 2 methodologies, according to the methodologies and guidelines of the International Panel on Climate Change (IPCC, 2006).
For accuracy, the enteric CH_4 emissions were calculated for an experimental a dairy cattle herd, of the the Research and Development Institute for Bovine Balotesti, during 2022-2024 period.
The gross energy intake (GE), digestible energy (DE), and methane conversion factor (Y_m) values for Tier 2, were calculated according to national reference values.
The enteric emissions were expressed as Gg CH_4 yr^{-1} and t CO_2-eq.
The methane emission was calculated based on equations 10.19 and 10.21 of the IPCC Guidelines (2006), using the formulas:

$$Emissions = EF_{(T)} \times N_{(T)}/10^6$$

where:
Emissions = methane emissions from enteric fermentation, Gg CH_4/yr^{-1};
$EF_{(T)}$ = emission factor for the defined livestock population, kg CH_4 /$head^{-1}/yr^{-1}$;
$N_{(T)}$ = the number of head of livestock species/category T in the country;
T = species/category of livestock.

$$EF = [GE \times (Y_m/100) \times 365]/55.65$$

where:
EF = emission factor for enteric fermentation (kg $CH_4/head^{-1}/yr^{-1}$);
GE = gross energy intake (MJ/head/year);
Y_m = methane conversion factor, per cent of gross energy in feed converted to methane;
The factor 55.65 (MJ/kg CH_4) is the energy content of methane.
To quantify methane emissions (t CO_2-eq) from dairy cattle enteric fermentation, the basic formula of the IPPC Tier 2 approach was used:

$$Emissions = N_{(T)} \times (EF_{enteric}/1000) \times GWP_{CH4}$$

where:
Emissions = methane emissions from enteric fermentation of dairy cattle, t CO_2-eq;
$N_{(T)}$ = the number of head of livestock species/category T in the country;
$EF_{(T)}$ = emission factor for the defined livestock population, kg $CH_4/head^{-1}/yr^{-1}$;
$GWP_{100\ CH4}$ = the global warming potential of CH_4 in 100 years;
1 kg CH_4= 25 kg CO_2-eq (Forster et al., 2007).

The gross energy intake (GE) was calculated based on the following equation:

$$GE\ (kcal/kg) = 5.72 \times GP + 9.5 \times GB + 4.79 \times CelB + 4.17 \times SEN$$ (Stoica, 1997)

where:
GE = gross energy intake;
GP = crude protein;
GB = crude fat;
CelB = crude fibres;
SEN = non-nitrate extractable substances.

The percentage of digestible energy (DE) of raw energy was calculated according to the following relation:

$$DE\ \% = (DE/GE) \times 100$$

To calculate the national values of Y_m the following equations (Cambra-Lopez, 2008) was used:

$$Y_m = -0.0038 \times (DE)^2 + 0.3501 \times DE - 0.811$$

Specific values for GE, DE, Y_m and EF were developed or were used from the literature (Table 1). For the Tier 1, emission factor (EF) was 99 kg/head/year for dairy cows in Eastern Europe with an average annual milk production of 2.550 kg assumed by IPCC, 2006.
For the Tier 2, emission factors (EF) of 81.18 kg/head/year and 87.35 kg/head/year were considered for dairy cows with an average body weight between 620-650 kg, average milk yield between 11.1-13.84 kg, and a diet (kg/head/day) consisting of 20-25 kg of maize silage, 4-4.5 kg quality alfalfa hay, and 2-2.5 kg concentrates.
Emission factor (EF) of 91.45 kg/head/year was considered for dairy cows with an average body weight of 650 kg, average milk yield 15.00 kg/head/day and a diet (kg/head/day) consists of 25-30 kg silage maize, 5 kg alfalfa

hay, and 6 kg concentrates. Emission factors (EF) of 95.23 kg/head/year, 115.40 kg/head/year, and 119.84 kg/head/year were considered for dairy cows with an average body weight of 650 kg, average milk yield between 16.30-16.48 kg/head/day and a diet (kg/head/day) consists of 28 kg silage maize, 7 kg alfalfa hay, and 6 kg concentrates.

Table 1. The values used for calculations of methane emission from dairy cows' enteric fermentation

Specification	Body Weight, kg	Average Milk Yield, kg/head/day	GE[1], MJ/head/ day	DE[2], MJ/head/ day	DE, %	Y_m[3], %	EF[4], kg/head/ year	Source
The value used for the number of dairy cattle reported by the National Institute of Statistics (NIS)								
Tier 1	-	-	-	-	-	-	99	IPPC, 2006
Tier 2	620	11.1	232.76	159.74	68.63	5.32	81.18	Popa et al., 2016
Tier 2	650	13.84	249.15	170.59	68.47	5.35	87.35	Popa et al., 2016
Tier 2	650	15.00	282.85	200.12	70.75	4.93	91.45	Own calculation
The value used for the number of dairy cattle from the Research and Development Institute for Bovine Balotesti								
Tier 2 - 2022	650	16.30	310.27	217.00	69.93	4.68	95.23	Own calculation
Tier 2 - 2023	650	16.37	312.34	211.93	67.85	5.85	119.84	Own calculation
Tier 2 - 2024	650	16.48	314.20	210.44	66.97	5.60	115.40	Own calculation

[1]GE = gros energy intake; [2]DE = digestible energy of gross energy; [3]Y_m = methane conversion factor of gross energy in feed converted to methane; [4] EF = emission factor.

RESULTS AND DISCUSSIONS

Total methane emissions estimated (Tier 1) from the Romanian dairy cattle sector between 2015-2024 are presented in Table 2. Applying the Tier 1 methods to Romanian's livestock, the obtained results revealed that the enteric CH_4 emissions decreased from 118.89 Gg CH_4/yr^{-1} (2.972.264 t CO_2-eq/year) to 106.88 Gg CH_4/yr^{-1} (2.672.010 t CO_2-eq/year) between 2015 and 2024. In contrast with our results, Sallaku et al. (2011) reported a higher value of 4.6943.4 t CO_2-eq/year obtained on a small herd of 22.580 dairy cows using the Tier 1 method.

Table 2. Summary of methane emissions estimated from the Romanian dairy cattle sector between 2015-2024

Years/Specification	Dairy cows, heads/year	Tier 1	CH_4 emission/head/year	
			Gg[2] CH_4/yr^{-1}	*t CO_2-eq*[3]
2015	1.200.915		118.89	2.972.264
2016	1.201.132		118.89	2.972.801
2017	1.168.975		115.72	2.893.213
2018	1.170.076		115.83	2.895.938
2019	1.147.399	EF[1]: 99	113.59	2.839.812
2020	1.130.638		111.93	2.798.329
2021	1.118.342		110.71	2.767.896
2022	1.104.215		109.31	2.732.932
2023	1.077.589		106.68	2.667.032
2024	1.079.600		106.88	2.672.010

[1]EF = emission factor; [2]Gg CH_4/yr^{-1} = gigagrams CH_4/year; [3]t CO_2-eq = tons CO_2 equivalent.

Summary of methane emissions estimated (Tier 2) from the Romanian dairy cattle sector between 2015-2024 are presented in Table 3.

Applying the Tier 2 methods using EF: 81.18 kg/head/year and EF: 87.35 kg/head/year, published by Popa et al. (2016), the enteric CH_4 emissions decreased gradually from reference year 2015 until the reference year 2024.

The decrease of CH_4 levels at national scale was attributed to the decrease in animal numbers.

Table 4 summarizes the methane emissions estimated from the Romanian dairy cattle between 2015-2024 using Tier 2 method, EF of 91.45 kg/head/year (own calculation).

Total methane emissions estimated for dairy cattle decreased from 109.82 Gg CH_4/yr^{-1} (2.7445.591 t CO2-eq/year) to 98.72 Gg CH_4/yr^{-1} (2.467.798 t CO2-eq/year) between year 2015 to year 2024.

Similarity, the decrease in methane emissions was attributed to the decrease of dairy cattle numbers.

Table 3. Summary of methane emissions estimated from the Romanian dairy cattle sector between 2015-2024

Years/ Specification	Dairy cow, head/year	Tier 2	CH_4 emission/head/year	
			Gg[2] CH_4/yr^{-1}	*t CO_2-eq*[3]
2015	1.200.915	EF[1]: 81.18	97.49	2.437.256
		EF: 87.35	104.89	2.622.498
2016	1.201.132	EF: 81.18	97.50	2.437.697
		EF: 87.35	104.91	2.622.972
2017	1.168.975	EF: 81.18	94.89	2.372.434
		EF: 87.35	102.10	2.552.749
2018	1.170.076	EF: 81.18	94.98	2.374.669
		EF: 87.35	102.20	2.555.153
2019	1.147.399	EF: 81.18	93.14	2.328.646
		EF: 87.35	100.22	2.505.632
2020	1.130.638	EF: 81.18	91.78	2.294.629
		EF: 87.35	98.76	2.469.030
2021	1.118.342	EF: 81.18	90.78	2.269.675
		EF: 87.35	97.68	2.442.179
2022	1.104.215	EF: 81.18	89.64	2.241.004
		EF: 87.35	96.45	2.411.329
2023	1.077.589	EF: 81.18	87.47	2.186.966
		EF: 87.35	94.12	2.353.184
2024	1.079.600	EF: 81.18	87.64	2.191.048
		EF: 87.35	94.30	2.357.576

[1]EF = emission factor; [2]Gg CH_4/yr^{-1} = gigagrams CH_4/year; [3]t CO_2-eq = tons CO_2 equivalent.

Table 4. Summary of methane emissions estimated from the Romanian dairy cattle sector between 2015-2024

Years/Specification	Dairy cow, head/year	Tier 2	CH_4 emission/head/year	
			Gg[2] CH_4/yr^{-1}	*t CO_2-eq*[3]
2015	1.200.915		109.82	2.745.591
2016	1.201.132		109.84	2.746.088
2017	1.168.975		106.90	2.672.569
2018	1.170.076		107.00	2.675.086
2019	1.147.399	EF[1]: 91.45	104.92	2.623.240
2020	1.130.638		103.39	2.584.921
2021	1.118.342		102.27	2.556.809
2022	1.104.215		100.98	2.524.511
2023	1.077.589		98.54	2.463.637
2024	1.079.600		98.72	2.468.235

[1]EF = emission factor; [2]Gg CH_4/yr^{-1} = gigagrams CH_4/year; [3]t CO_2-eq = tons CO_2 equivalent.

Table 5 presents a summary of methane emissions estimated from a dairy cows' experimental herd between 2022 and 2024. The CH_4 emissions from enteric fermentation were calculated first by the Tier 1 method, resulting in an estimated amount of 210.37 *t CO_2-eq* in year 2022, 207.90 *t CO_2-eq* in year 2023, and 198.00 *t CO_2-eq* in year 2024 (0.008415 Gg CH_4/yr^{-1} to 0.007920 Gg CH_4/yr^{-1}).

Following the Tier 2 approach, the CH_4 emission/head/year were of 202.36 *t CO_2-eq* in year 2022, 251.66 *t CO_2-eq* in year 2023, and of 230.80 *t CO_2-eq* in year 2024, which were higher than the default value for EF Tier 1 from IPCC (2006).

Results are in line with those of (Popa et al., 2021), which showed that feed diets have a significant influence on enteric CH_4 emission.

Also, diet supplemented with 0.2 l/head/day rapeseed oil and 0.1 l/head/day sunflower oil showed a CH_4 emissions values of 1.41 kg CH_4/year (35.30 t CO_2 x 10^{-9}/year) and 1.39 kg CH_4/year (34.75 t CO_2 x 10^{-9}/year) in a herd of 29 Montbeliarde dairy cows in different lactation stages (Popa et al., 2022). Furthermore, in dairy buffaloes, the methane emissions estimates from enteric fermentation were between 0.002244 - 0.004280 Gg CH_4/yr^{-1} during 2014-2017 (Popa et al., 2017), values that are lower than those for dairy cattle.

Table 5. Summary of methane emissions estimated from the Romanian Black and Spotted dairy cows

Years/Specification	Dairy cows, heads/year	Tier 1	CH_4 emission/head/year	
			Gg[2] CH_4/yr^{-1}	*t CO_2-eq*[3]
2022	85		0.008415	210.37
2023	84	EF[1]:99	0.008316	207.90
2024	80		0.007920	198.00
		Tier 2		
2022	85	EF:95.23	0.008094	202.36
2023	84	EF:119.84	0.010066	251.66
2024	80	EF:115.40	0.009232	230.80

[1]EF = emission factor; [2]Gg CH_4/yr^{-1} = gigagrams CH_4/year; [3]t CO_2-eq = tons CO_2 equivalent.

The paper presents the *status-quo* of the Romanian dairy sector and the resulting GHGs. We admit some limitations of the study, such as the lack of determinations of the current feed energy content and reporting data based on national averages, rather than on farm specific indicators (milk yield, reproduction performances, feed production, etc.). However, there is a strong body of literature showing that the process of rumen methanogenesis is important in achieving a regional strategy to further reduce methane gas emissions.

To decrease GHG emissions from the sector, enteric methane emissions from dairy cattle should be decreased without reducing livestock numbers, rather than identifying management and nutritional solutions, such as various feed additives.

The ways to reduce greenhouse gas emissions need to be adapted to specific local targets and conditions, by improving feed digestibility, forage quality, and diet composition; with an aim to improve the selection strategies and health of the animals; crop management and manure management (Marin et al., 2020). Furthermore, dietary optimization and the use of feed additives can reduce enteric CH_4 emissions directly, whereas selective breeding could have effects in the future generations (Kroliczewska et al., 2023), given the long productive cycle of cattle, with between-generation intervals of 4.5-5 years. Moreover, in cattle the production of ruminal methane is directly proportional to the digestibility of feed ingested. Feed with high digestibility produces less methane per unit of caloric energy consumed, than feed with lower digestibility (Yayli & Kilic, 2020). Sauvant & Giger-Reverdin (2009) shown that diet composition and intake are representing the main factors affecting CH_4 production by ruminants, with forages that have higher structural carbohydrates producing more CH_4 than those fed mixed diets containing higher levels of non-structural carbohydrates per unit of fermented material at the rumen level.

CONCLUSIONS

Total methane emissions estimated for the Romanian dairy cattle decreased during the period 2015-2024. The decrease in methane emissions was attributed to the decrease in the number of dairy cattle at national level.

The results obtained revealed that Romanian estimates of enteric CH_4 emissions differ when calculated using the IPCC Tier 2 methodology, compared to the IPCC Tier 1 methodology. As a result, more research is needed to identify the most accurate method between the two.

Strategies to reduce enteric methane emissions in combination with increasing animal performance (without reducing animal numbers) could lead to a reduced intensity of enteric CH_4 emissions.

The current results can provide information on the *status quo* of the dairy industry in terms of environmental footprint, as well as baseline information to develop appropriate future strategies to reduce the carbon footprint of the cattle sector numbers.

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EFFECT OF DIETARY INCLUSION OF QUINOA SEED ON PRODUCTIVITY, EGG QUALITY AND INTERNAL ORGAN TRAITS IN QUAILS

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Abstract

This study examined the effects of dietary quinoa seed (Chenopodium quinoa Willd.) supplementation on production performance, egg quality and internal organ traits in Japanese quails (Coturnix coturnix japonica). A total of 90 six-week-old quails were randomly divided into three groups: a control group and two treatment groups receiving diets supplemented with 5% and 10% quinoa seeds, respectively. Over a 8-week trial, key performance indicators were evaluated, including egg weight, feed conversion ratio, internal organ development, and egg quality traits. Results indicated that 5% quinoa inclusion significantly improved egg weight, feed conversion ratio, and small intestine development (P<0.05). Additionally, both quinoa-supplemented groups showed enhanced yolk pigmentation, with increased yellowness (b) and lightness (L), but a decrease in yolk index. No adverse effects were observed on overall egg production, feed intake, or internal organ weights (P>0.05). These findings suggest that quinoa, particularly at 5% inclusion, is a valuable feed additive for enhancing quail productivity and egg quality without compromising health or performance, supporting its use as a functional and sustainable alternative ingredient in poultry nutrition.

Key words*: egg quality traits, Japanese quails, quinoa seed, production performance.*

INTRODUCTION

Recent years have witnessed growing interest in the use of alternative feed ingredients in poultry nutrition. driven by the need for sustainable cost-effective and nutritionally rich options. One such promising alternative is *Chenopodium quinoa* (quinoa), a pseudocereal renowned for its rich nutrient composition, including high-quality protein, essential amino acids, unsaturated fatty acids, vitamins. minerals and antioxidants (Vega-Gálvez et al., 2010). Native to the Andean regions of Bolivia, Chile and Peru. quinoa is also cultivated in southern parts of Iran as a potential protein source in animal feeding systems (Amiri et al., 2021). The interest in utilizing alternative protein sources like quinoa in poultry diets has grown substantially. Quinoa stands out due to its richness in essential amino acids such as lysine and methionine and its notable content of water-soluble vitamins including riboflavin, thiamine and niacin. It is also a good source of minerals such as manganese, iron, potassium, copper. zinc and phosphorus (Amiri et al., 2021). Compared to conventional cereals such as corn, barley, wheat and oats. Quinoa contains higher levels of metabolizable energy, calcium, iron, phosphorus and vitamins B and E (Vilche et al., 2003; Konishi et al., 2004). Despite these advantages quinoa also contains certain anti-nutritional compounds such as saponins, phytates, and trypsin inhibitors, which may negatively affect nutrient absorption in monogastric animals like poultry (Oboh & Elusiyan, 2007). Therefore, its inclusion in poultry diets must be approached with consideration for these factors. Studies have demonstrated that applying various processing techniques to quinoa can enhance its digestibility, thereby promoting better nutrient absorption and increased body weight in poultry (Eassawy et al., 2016). Furthermore, quinoa seed extract contains high levels of phenolic and antioxidant compounds, which are associated with improved growth performance and feed efficiency. The incorporation of bioactive-rich functional ingredients like quinoa into poultry diets has also been shown to enhance various egg quality traits - such as yolk pigmentation, shell strength, and albumen height - primarily due to the presence of beneficial compounds like flavonoids and saponins (González et al., 2012; Repo-Carrasco-Valencia & Serna, 2011). Moreover,

dietary modifications can significantly affect avian reproductive physiology by influencing ovarian development, follicular growth, and hormonal regulation (Saki et al., 2011). While studies on broilers have shown that quinoa seed supplementation can enhance body weight gain and improve feed conversion ratios (Nokandi et al., 2021), research specifically focusing on the effects of quinoa inclusion on laying performance, egg quality, and reproductive organ development in quails (*Coturnix coturnix japonica*) - a species recognized for its high egg production and efficient feed utilization - remains limited. Accordingly, this study aims to assess the effects of dietary quinoa seed supplementation on productivity egg quality and internal organ traits in laying quails. The results are anticipated to address significant gaps in the existing literature and provide evidence-based insights to enhance the sustainability and efficiency of quail nutrition strategies.

MATERIALS AND METHODS

This study was conducted following ethical guidelines and was approved by the Dicle University Experimental Animal Ethics Committee (DÜHADEK) under protocol number 2023/21.

This experiment was carried out at the Poultry Research and Application Unit of Dicle University. Ninety six-week-old Japanese quails *(Coturnix coturnix japonica),* housed in cages at a ratio of one male to two females, were randomly assigned to three dietary treatment groups, each consisting of 10 replicates. The study spanned a 10-week period. The experimental design included: (1) a control group fed a standard basal diet, and (2) two treatment groups receiving the same basal diet supplemented with either 5% or 10% quinoa seed. All diets were formulated to meet the nutritional requirements specified by the National Research Council (NRC, 1994), ensuring a consistent basal composition across all groups (Table 1). The experimental diets were formulated to ensure comparable levels of protein and energy. The proximate composition of the diets was determined using standard analytical procedures as outlined by the Association of Official Analytical Chemists (AOAC, 2005). Nutrient contents. including crude protein, crude fat, crude ash, fiber, calcium and phosphorus were calculated to meet the nutritional requirements of laying quails.

Table 1. Ingredients and chemical composition of the experimental diets

Ingredients	**%**		
	Quinoa 0 (Control)	Quinoa 5	Quinoa 10
Sunflower oil	2.20	2.00	2.00
Maize	58.00	54.20	50.20
Soybean Meal (44%. CP)	22.00	21.00	22.00
Sunflower Meal (32%. CP)	8.00	8.00	8.00
Quinoa seeds	-	5.00	10.00
Dicalcium Phosphate[a]	2.00	2.00	2.00
Limestone	7.00	7.00	7.00
DL-Methionine	0.15	0.15	0.15
NaCl	0.30	0.30	0.30
Vitamin and Mineral Premix[b]	0.25	0.25	0.25
Items	**Analyzed values, %**		
Dry Matter	90.3	90.3	90.3
Crude Protein	17.1	17.1	17.1
Ether extract	4.12	4.12	4.10
Crude Ash	12.12	12.14	12.15
Crude Fiber	3.90	3.89	3.87
Items	**Calculated values, %**		
Metabolizable energy (ME). kcal/kg	2750	2750	2750
Calcium	3.24	3.24	3.24
Available Phosphorus	0.44	0.44	0.44
Na	0.17	0.17	0.17
Cl	0.23	0.23	0.23
Methionine + Cysteine	0.70	0.70	0.70
Lysine	0.87	0.87	0.87
Threonine	0.61	0.59	0.59
Tryptophan	0.23	0.22	0.22
Linoleic acid	2.17	2.16	2.16
Electrolyte Balance (mEq/kg DM)	194	192	192

[a]Composition: 240 g Ca and 17.5 g P/kg; [b]Vit+Min. Mineral mixture provides the following nutrients per kg of diet: vitamin A: 12.000.000 IU; vitamin D3: 2.500.000 IU; vitamin E: 30 ppm; vitamin K3: 4.000 ppm; vitamin B1: 3.000 ppm; vitamin B2: 7.000 mg; vitamin B12: 5.000 ppm; vitamin C: 50.000 ppm; Biotin: 45 ppm; folic acid: 1.000 ppm; Fe

The control group was fed a basal diet without quinoa supplementation. whereas the treatment groups received diets supplemented with 5% and 10% quinoa seeds respectively. The experimental period lasted for eight weeks, during which relevant data were systematically recorded. The study evaluated parameters including egg quality, feed intake, feed conversion ratio, internal organ weights.

Throughout the experimental period a lighting schedule of 16 hours light and 8 hours dark (16L:8D) was maintained. Feeders were manually refilled daily and eggs were collected each morning. At the onset of the trial, hens were individually weighed and allocated to cages based on similar body weights and egg production levels to ensure uniformity across

treatment groups. Feed intake, egg production and egg weight were recorded daily while feed conversion ratio and other performance parameters were calculated on a weekly basis.

During the analysis phase. a total of 30 eggs were evaluated each week with one egg randomly selected from each cage. Eggs were collected from each group over two consecutive mornings. stored overnight at 4°C and subsequently subjected to both external and internal quality assessments. By the conclusion of the experiment. a total of 240 eggs had been analyzed.

Specific gravity was determined by weighing each egg after a one-day storage period under ambient conditions, followed by immersion in water at 22°C and subsequent reweighing in accordance with the method outlined by Hempe et al. (1988). Yolk colour was evaluated using a digital colorimeter (Minolta CR-300) with measurements expressed as L, a, and b values. Albumen height was measured using a digital micrometer and subsequently used to calculate the albumen index using the formula: [albumen height (mm) / ((albumen length (mm) + albumen width (mm)) / 2)] × 100.

At the end of the experiment, 10 female quails from each experimental group (one bird per replicate) were randomly selected to determine internal organ weights. The birds were euthanized in accordance with ethical guidelines, and careful dissection was performed starting from the breastbone to open the abdominal cavity. Non-carcass internal organs such as the liver, heart, spleen, pancreas, kidneys, and abdominal fat tissue were carefully excised. After removal, any excess fluids and residues on the organs were cleaned to prevent contamination before weighing. Each organ was weighed immediately using a precision balance. Relative internal organ weights were calculated using the formula: "Organ Weight (g) / Live Body Weight (g) × 100".

Data were subjected to one-way analysis of variance (ANOVA) using SPSS software (version 18.0; IBM Corp. Armonk. NY. USA). When significant differences were detected. treatment means were compared using Tukey's multiple comparison test at a significance level of $P<0.05$. Additionally. regression analysis was conducted to evaluate linear. quadratic. and cubic trends in response to varying levels of mealworm inclusion.

RESULTS AND DISCUSSIONS

Table 2 evaluates the effects of dietary quinoa seed inclusion at 0%, 5%, and 10% on the productivity performance of Japanese quails. The 5% quinoa group recorded significantly higher egg weights compared to the control and 10% quinoa groups ($P< 0.01$). In addition, both 5% and 10% quinoa groups had improved FCR compared to the control ($P<0.05$). However, egg Production and Daily Feed Intake: These parameters did not significantly differ across groups ($P > 0.05$). The improvement in egg weight and FCR with 5% quinoa aligns with the nutrient-rich profile of quinoa, particularly its high-quality protein and balanced amino acid content (Amiri et al., 2021). Quinoa is also rich in essential amino acids such as lysine and methionine, which are critical for egg formation and overall productivity (Vega-Gálvez et al., 2010). The enhanced FCR indicates better nutrient utilization, likely influenced by quinoa's bioactive compounds including flavonoids and antioxidants. These components have been shown to promote gut health and improve metabolic efficiency, as supported by Nokandi et al. (2021) who reported improved performance in broilers fed quinoa-based diets. Interestingly, the 10% inclusion did not yield further improvements and slightly reduced egg weight compared to 5%, suggesting a threshold effect beyond which no additional benefits occur - possibly due to anti-nutritional factors such as saponins and phytates, known to impair nutrient absorption in monogastrics (Oboh & Elusiyan, 2007). This highlights the importance of optimal inclusion levels to balance benefits with potential drawbacks. Moreover, the non-significant differences in egg production and feed intake across groups support previous findings that quinoa does not negatively affect laying rate or appetite (Naïmatî et al., 2022), thus maintaining reproductive performance while enhancing efficiency. Quinoa inclusion at 5% appears to be optimal, enhancing egg weight and feed efficiency without compromising overall productivity. This supports quinoa's role as a sustainable and functional ingredient in poultry

nutrition, corroborated by previous research on its positive impacts on performance and intestinal health (Eassawy et al., 2016; González et al., 2012).

The findings in Table 3 illustrate the influence of different dietary levels of quinoa seeds on the egg quality traits of Japanese quails. Notably, while most traits such as shell weight, shell thickness, shape index, and albumen index were statistically non-significant ($P>0.05$), yolk-related characteristics and colorimetric values revealed meaningful variation among treatments. The yolk index significantly decreased with quinoa supplementation, with the control group showing the highest value compared to the 5% and 10% quinoa groups ($P < 0.001$). This finding is contrary to expectations based on the antioxidant and lipid profile of quinoa, which are generally associated with yolk structural integrity (González et al., 2012; Repo-Carrasco-Valencia & Serna, 2011). The reduction may stem from structural changes in yolk composition due to quinoa's saponin content, which has been known to influence nutrient absorption (Oboh & Elusiyan, 2007). In terms of yolk coloration, the lightness (L), redness (a), and yellowness (b) values were all significantly altered by quinoa inclusion. The 5% quinoa group exhibited the highest L value indicating lighter yolks compared to control and 10% ($P<0.01$). Redness (a) was significantly lower in the 5% group than in the control ($P <0.05$), whereas yellowness (b*) was significantly higher in both quinoa groups compared to the control ($P<0.01$). These results align with previous reports that flavonoids and carotenoids in quinoa enhance yolk pigmentation, particularly increasing b values indicative of a deeper yellow hue (Vilche et al., 2003; Amiri et al., 2021).

Other quality traits such as specific gravity and shell characteristics showed no significant changes, suggesting that quinoa supplementation at up to 10% does not compromise shell integrity. This stability is crucial for commercial egg production, where shell robustness affects transport and storage quality (Hempe et al., 1988). Overall, these results support earlier research demonstrating the potential of quinoa as a natural pigment enhancer and nutrient-dense feed additive in poultry diets (Eassawy et al., 2016; Nokandi et al., 2021). However, the decline in yolk index prompts further study into the biochemical interactions of quinoa components within avian physiology.

Table 4 presents the effects of different dietary quinoa seed levels on the relative weights of non-carcass internal organs in female Japanese quails. Among the measured organs, only the small intestine showed a statistically significant response ($P<0.01$) to quinoa inclusion, whereas the heart, liver, abdominal fat, stomach, gizzard, cecum, and large intestine weights were unaffected ($P > 0.05$). The small intestine weight was significantly higher in the 5% and 10% quinoa groups compared to the control, suggesting enhanced intestinal development with quinoa supplementation. This result is in line with findings from Eassawy et al. (2016), who reported improved intestinal morphology and nutrient absorption in poultry following inclusion of antioxidant-rich feed components like quinoa. The enhanced gut development may be attributed to quinoa's bioactive compounds, including polyphenols and flavonoids, known to promote gut health and modulate microbiota composition (Repo-Carrasco-Valencia & Serna, 2011). The lack of significant change in liver and abdominal fat weights indicates that quinoa supplementation does not adversely affect lipid metabolism or fat deposition. This is consistent with findings by Nokandi et al. (2021), where dietary quinoa did not alter fat pad weights in broilers, likely due to its favorable fatty acid composition and presence of saponins, which can reduce lipid absorption. Interestingly, the weights of digestive organs such as the stomach and gizzard were also unaffected. This suggests that quinoa inclusion at the tested levels does not impair digestive organ development or function. Similarly, unchanged cecum and large intestine weights point to the maintenance of hindgut health and fermentation capacity, supporting the use of quinoa as a gut-friendly feed ingredient. Collectively, the results indicate that dietary quinoa, particularly at 5%, promotes intestinal development without negatively impacting other internal organs. These findings further support quinoa's potential as a functional feed additive that can enhance nutrient utilization in quails without

compromising visceral health, aligning with previous reports emphasizing quinoa's high digestibility and nutritional quality (Amiri et al., 2021; González et al., 2012).

Table 2. Productivity Performance of Quails Fed Diets Containing Varying Levels of Quinoa Seed

Measurements	Quinova levels (%)					Effects[1]		
	0 (Control)	5	10	SEM[2]	*P-value*	L	Q	C
Egg Weight (EW), g	13.1[b]	13.6[a]	13.3[ab]	0.060	0.002	NS	**	**
Egg Production. %	87.6	85.3	82.2	1.340	0.248	NS	NS	NS
Feed Intake (FI), g/quail/day	42.0	41.9	40.8	0.380	0.338	NS	NS	NS
Feed Conversion Rate (FI/EW)	3.22[a]	3.08[b]	3.06[b]	0.029	0.049	**	**	**

[a,b]Means within each period with different superscript letters are significantly different (P < 0.05)
[1]Effects: L=Linear; Q= quadratic; C=cubic; [2]SEM = pooled standard error of the mean; NS = not significant.

Table 3. Egg quality traits of quails Fed Diets Containing Varying Levels of Quinoa Seed

Measurements	Quinova levels (%)					Effects[1]		
	0 (Control)	5	10	SEM[2]	P	L	Q	C
Specific Gravity	1.065	1.065	1.065	0.0004	0.974	NS	NS	NS
Shell weight, g	1.458	1.526	1.458	0.015	0.117	NS	NS	NS
Egg Shell Rate	10.87	10.95	10.85	0.106	0.919	NS	NS	NS
Shell Thickness, mm	0.334	0.321	0.309	0.008	0.515	NS	NS	NS
Shape Index	77.3	77.0	77.8	0.274	0.452	NS	NS	NS
Albumen Index	19.06	19.71	19.44	0.169	0.307	NS	NS	NS
Yolk Index	49.28[a]	45.41[b]	44.41[b]	0.516	<0.001	**	**	**
L*	44.26[b]	46.65[a]	45.53[ab]	0.331	0.011	NS	**	**
a**	22.8[a]	21.6[b]	22.01[b]	0.201	0.029	**	**	**
b****	32.5[b]	34.5[a]	33.8[a]	0.317	0.009	**	**	**

[a,b]Means within [each] period with different superscript letters are significantly different (P < 0.05)
[1]Effects: L=Linear; Q= quadratic; C=cubic; [2]SEM = pooled standard error of the mean; NS = not significant;
L*: lightness; a**: redness; b***: yellowness.

Table 4. The relative weight of non-carcass part and internal organ traits of female quails fed quinoa seed-based diets

Weights of Internal Organs, %	Quinova levels (%)			SEM[2]	P	Effects[1]		
	0 (Control)	5	10			L	Q	C
Heart Weight	0.665	0.658	0.572	0.041	0.615	NS	NS	NS
Liver Weight	2.352	1.821	2.101	0.113	0.161	NS	NS	NS
Abdominal Fat Pad	2.072	1.624	2.152	0.220	0.594	NS	NS	NS
Proventriculus	0.222	0.260	0.272	0.021	0.619	NS	NS	NS
Gizzard	1.821	1.548	1.971	0.099	0.214	NS	NS	NS
Small Intestine	1.927[b]	2.314[a]	2.304[a]	0.064	0.010*	NS	NS	NS
Cecum	0.457	0.278	0.381	0.075	0.150	NS	NS	NS
Large Intestine	0.135	0.268	0.168	0.045	0.090	NS	NS	NS

[a,b]Means within each period with different superscript letters are significantly different (P < 0.05)
[1]Effects: L=Linear; Q= quadratic; C=cubic; [2]SEM = pooled standard error of the mean; [3]NS = not significant.

CONCLUSIONS

This study demonstrated that dietary inclusion of quinoa seeds at 5% and 10% levels in quail diets can enhance certain productive and physiological parameters without adverse effects. Specifically, 5% quinoa supplementation significantly improved egg weight, feed conversion ratio, and small intestine development. Additionally, both quinoa levels enhanced yolk pigmentation, particularly increasing yellowness (b), while maintaining shell quality and other internal organ traits. These findings suggest that quinoa seeds represent a valuable, sustainable alternative protein and functional ingredient in quail nutrition, contributing positively to productivity and egg quality.

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SUSTAINABLE NUTRITIONAL SOLUTIONS FOR ANIMAL PRODUCTION: OPTIMISING NUTRITION TO REDUCE POLLUTION

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Abstract

The contribution of animal production to environmental pollution is a pressing global issue. This study aims to evaluate the chemical composition of selected feeds and explore the relationship between feed composition and pollutant emissions generated by animals. Chemical analyses of 10 feed samples assessed parameters such as protein, cellulose, fat, and nitrogen-free extractive contents. The goal was to identify some connection with pollutant emissions and propose nutritional strategies to reduce emissions, improve nutrient utilisation, and promote the sustainable management of animal resources. The results revealed potential links between feed composition and different emissions from animals with considerable environmental impact. High-protein feeds were associated with elevated nitrogen residues, while feeds with greater digestibility showed potential for reduced pollutant emissions. Future efforts should focus on adjusting feed composition and integrating optimised feed formulations to support a sustainable approach to animal nutrition, and reducing pollution from livestock production systems, benefiting both the environment and public health.

Key words*: environment, feed composition, livestock, pollution.*

INTRODUCTION

Industrial and technological developments, needed to support a growing population, have generated different pollutants, adversely impacting ecosystems. The destabilisation of the natural environments often results from antropogenic activities, whether conscious or inadvertent, which introduce disruptive factors that interact with various ecosystem components. Agriculture, particularly animal production, considerably contributes to environmental pollution through greenhouse gas emissions or manure rich in nitrogen and phosphorus, which degrade soil and water quality (U.S. EPA, 2019; Ukaogo et al., 2020). Due to their dynamic behaviour, bioaccumulation potential, and inherent toxicity, pollutants pose a serious threat to all environmental elements. Consequently, phased monitoring of their impact, along the trophic cycle – from soil and agricultural crops to organisms and animal–derived products – is required for understanding and mitigating their cumulative effects (Matei et al., 2024b). Recent changes in pollution levels and population consumption trends, characterised by an increasing demand for animal products, underscore the close connection between animal husbandry and the environment. This dynamic highlight the importance of producing safe (Postolache et al., 2020; Lăpușneanu et al., 2025), high-quality outputs while ensuring animal welfare and implementing effective strategies to minimize pollution's environmental impact and preserve ecological balance (Mitchell, 2007). Pollution stemming from animal production has become a major concern, particularly due to intensive practices and diversification of production methods. While the interaction between animals and the environment is unavoidable (Postolache et al., 2015), animal husbandry paradoxically represents a major source of pollution, contributing through waste, gaseous emissions, and chemical residues from current treatment practices applied to livestock (Dumitraș, 2008).

The polluting potential of animal waste is determined by the substances present in manure, which, when exceeding the environment's tolerance levels, contribute to its gradual degradation. According to the U.S. EPA (2019), excess phosphorus and nitrogen in animal manure are among the primary factors negatively

affecting the environment. Among the residual constituents, Neumeier & Mitloehner (2013) emphasise emissions with considerable environmental impacts, including greenhouse gases, hydrogen sulphide, ammonia, or volatile organic compounds. In practice, ammonia, methane, and nitrous oxide are the principal pollutants linked to animal production; these pollutants have direct implications for climate change, air quality, and water pollution. Methane (CH_4) has been classified as a main pollutant due to its major contribution to the greenhouse effect and global warming (IPCC, 2013). Other gases contributing to the greenhouse effect are carbon dioxide (CO_2), nitrous oxide (N_2O), and fluorinated gases (U.S. EPA, 2019; IPCC, 2014). From a nutritional perspective, the feed administered to animals directly influences their health and productivity (Rațu et al., 2021). The ideal functioning of the animal body and its production performance are closely tied to the energy density of the feed, the balance of nutrients, and their biologically active components assimilated from the feed (Coșman et al., 2018). Research on feed efficiency has demonstrated that factors such as dry matter digestibility, protein and fat content, and feed safety are determinants for nutrient absorption (Liu et al., 2018; Gallo et al., 2020; Pu et al., 2021; Mwaniki et al., 2021). The chemical and biological characteristics of feeds, such as digestibility and nutrient composition, are essential for optimizing growth, minimizing overfeeding, and reducing environmental impact. A balanced diet, associated with feed availability and nutritional profile knowledge, contributes to reducing waste, reducing polluting emissions (Neumeier & Mitloehner, 2013) and limiting economic losses. However, while well-formulated feeds enhance animal health and productivity, they can also serve as vectors for contaminants, impacting both animal health and the environment (Matei & Pop, 2022; Lăpușneanu et al., 2024). Contaminants such as mineral oil hydrocarbons (MOSH/MOAH), pesticides, or heavy metals can interfere with animal health and affect the ecosystem's balance (Ciobanu et al., 2020; Boișteanu et al., 2024). A serious concern is the transfer of these pollutants from feed to animals and, subsequently, to products intended for human consumption (Matei et al., 2024a). Different studies (Jaspers et al., 2014; Ali et al., 2019) highlighted the mechanisms of pollutant bioaccumulation and transfer, underscoring the pivotal role of feed composition and nutritional management in mitigating their impact on health and the environment.

In this context, the work aims to analyze the chemical composition of certain feeds and investigate the connections between it and polluting emissions linked to animal production. The main objective is to identify sustainable nutritional solutions and to create strategies that contribute to reducing the environmental impact by improving nutrition and resource efficiency. These initiatives are intended to provide a deeper understanding of the role of animal production in pollution and to support the implementation of sustainable farm practices, with positive effects on both the environment and public health.

MATERIALS AND METHODS

The analyses carried out aimed to determine the raw chemical composition of the feeds while establishing their relationship with the polluting emissions associated with animal production. The investigations aimed to assess the impact of technological conditions on the pollution linked to animal production and to identify solutions for optimizing nutrition and promoting the efficient use of available resources.

Sample Collection

The research material was represented by 10 samples of animal feed collected from a livestock farm. These samples included different types of feed, such as fresh, dried, preserved, concentrated, and mixed. The samples were collected following the SR EN ISO 6497:2005 standard and the provisions of Regulation (EC) 152/2009–Annex I to make sure they were accurate and fair. The final samples weighed between 1.5 and 3.0 kg and were prepared for analysis following the rules to keep the integrity of chemical composition. The way the samples were prepared followed the guidelines outlined in SR EN ISO 6498:2012 and the provisions of Regulation (EC) 152/2009–Annex II.

Sample Processing

To determine the crude chemical composition, the samples were dried, ground, and homogenized using methods that preserved the integrity of the compounds and prevented the denaturation of essential components. Bulk forages were initially manually cut to 1-2 cm sizes, while fresh and pickled feed were pre-dried at 60ºC, to reach 8-12% moisture content. Afterward, all the samples were ground into a fine powder using the Grindomix GM 200 lab mill. For the concentrated feed samples, they were directly ground into a fine powder using the same Grindomix GM 200 mill.

Chemical Analysis

Chemical analyses were done according to standardized and internationally recognized methods, with literature additional adaptations (Matei & Pop, 2023). The crude chemical composition was performed using the modified Weende method, as described in Annex III of Regulations (EC) 152/2009. These analyses followed the methods set by national and international standards for each constituent: SR ISO 6496:2001; SR EN ISO 6540:2021; SR ISO 712:2010 (moisture); SR EN ISO 2171:2010 (ash); SR EN ISO 5983-1:2006/AC:2019 (crude protein); SR ISO 6492:2001/AC:2016 (ether extract/fats); SR EN ISO 6865:2002/AC:2017 (crude fiber).

Processing and Data Interpretation

The quality of the analyses was ensured by using reference standards for equipment calibration and method validation. Data reproducibility was confirmed through replicate analyses for each sample, verifying the consistency of the results. For statistical processing, primary estimators of position (mean) and variation (standard deviation, coefficient of variation) were calculated. The interpretation of the results was achieved by correlating them according to the type of samples and the selected variables, being discussed by referring to literatura data.

RESULTS AND DISCUSSIONS

In this study, feed samples were analyzed to determine their raw chemical composition, such as the content of organic and inorganic substances; Table 1 summarizes the average results on crude chemical composition. The results were compared with reference values reported in the literature and contextualized with finding from previous similar research.

To investigate the relationship between the chemical composition of feed and the potential for polluting emissions from animal production, the study hypothesized that a balanced ration – defined as a mixture of feeds rich in energy substances, with a moderate protein and cellulose content – can optimize the utilization of nutritional resources and reducing the polluting impact, by improving feeding efficiency and reducing emissions of environmentally harmful gases.

The ration includes the essential parameters that influence the nutritional balance of animal nutrition, based on various feed sources with different nutritional profiles (dry matter DM, ash, crude protein CP, ether extract EE, crude fiber CF, non-nitrogenous extractive substances NES). These parameters are analyzed through the coefficient of variation (CV, %), which reflects the variability of each nutrient and provides insights into their consistency across the ration (Figure 1).

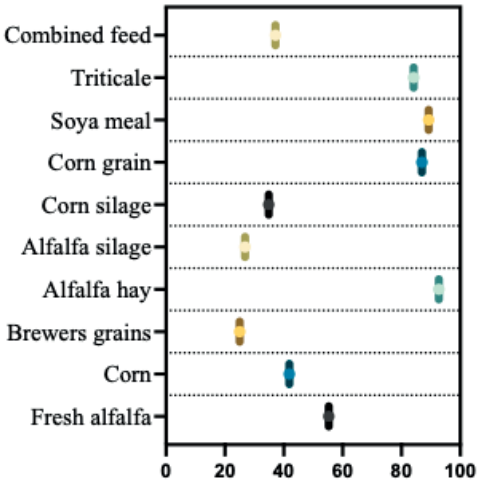


Figure 1. Dry matter content of feed (%) – the main source of nutrients available to animals

The ration is formulated using a mix of green feeds, wet roughage and hay, each adding different content of CP, NES and CF (Figure 2). Soybean meal, its high crude protein content (54.58% DM) is an important contributor to the

protein intake of the ration. Meanwhile, corn grain, rich in NES (81.72% DM), fulfills the energy and growth requirements of the animals. Additionally, hay, silage and brewer's grains supply considerable amounts of CF, important for maintaining an optimal digestive process in livestock.

Table 1. Average data and variability of chemical composition of feeds

Sample	Mean	DM[1]	Ash	OM[2]	EE[3]	CP[4]	CF[5]	NES[6]	NES / CF
		%	% DM						
Fresh alfalfa	$\bar{x}\pm s_{\bar{x}}$	55.32±0.02	13.16±0.07	86.82±0.06	2.35±0.13	28.59±0.07	21.74±0.15	34.14±0.12	1.57
	CV (%)	0.11	1.22	-	12.77	0.59	1.59	0.84	-
Corn	$\bar{x}\pm s_{\bar{x}}$	41.89±0.02	2.84±0.06	97.14±0.06	2.54±0.16	7.92±0.06	19.63±0.05	67.05±0.26	3.42
	CV (%)	0.10	5.38	-	14.66	1.74	0.62	0.88	-
Brewer's grains	$\bar{x}\pm s_{\bar{x}}$	24.99±0.05	4.02±0.03	95.95±0.03	8.97±0.17	34.14±0.03	15.14±0.12	37.70±0.18	2.49
	CV (%)	0.01	2.07	-	4.27	0.24	1.90	1.08	-
Alfalfa hay	$\bar{x}\pm s_{\bar{x}}$	92.71±0.02	13.22±0.09	86.75±0.09	1.81±0.07	21.92±0.10	28.22±0.11	34.80±0.08	1.23
	CV (%)	0.70	1.67	-	8.78	1.08	0.88	0.57	-
Alfalfa silage	$\bar{x}\pm s_{\bar{x}}$	26.85±0.02	13.52±0.14	86.45±0.14	2.04±0.06	14.07±0.06	36.16±0.10	34.18±0.21	0.95
	CV (%)	0.07	2.40	-	7.38	0.99	0.62	1.39	-
Corn silage	$\bar{x}\pm s_{\bar{x}}$	34.92±0.01	3.31±0.03	96.67±0.03	3.63±0.09	8.77±0.10	18.42±0.09	65.85±0.21	3.57
	CV (%)	0.06	2.40	-	5.71	2.63	1.14	0.72	-
Corn grain	$\bar{x}\pm s_{\bar{x}}$	86.95±0.07	1.32±0.01	98.66±0.01	4.30±0.07	10.66±0.13	1.98±0.03	81.72±0.16	41.27
	CV (%)	1.34	2.08	-	3.91	2.73	3.53	0.44	-
Soya meal	$\bar{x}\pm s_{\bar{x}}$	89.26±0.03	7.28±0.01	92.70±0.08	1.22±0.07	54.58±0.11	3.83±0.07	33.07±0.19	8.63
	CV (%)	0.79	0.34	-	13.90	0.47	4.53	1.33	-
Triticale	$\bar{x}\pm s_{\bar{x}}$	84.14±0.01	2.25±0.02	97.73±0.03	1.63±0.12	15.37±0.04	3.06±0.04	77.67±0.11	25.38
	CV (%)	0.19	2.81	-	17.46	0.65	3.42	0.34	-
Combined feed	$\bar{x}\pm s_{\bar{x}}$	37.16±0.01	4.31±0.04	95.66±0.04	2.92±0.07	10.10±0.07	18.35±0.04	64.29±0.12	3.5
	CV (%)	0.05	2.21	-	5.96	1.61	0.48	0.41	-

[1]DM=dry matter; [2]OM=organic matter; [3]EE=ether extract; [4]CP=crude protein; [5]CF=crude fiber; [6]NES=non-nitrogenous extractives substances.

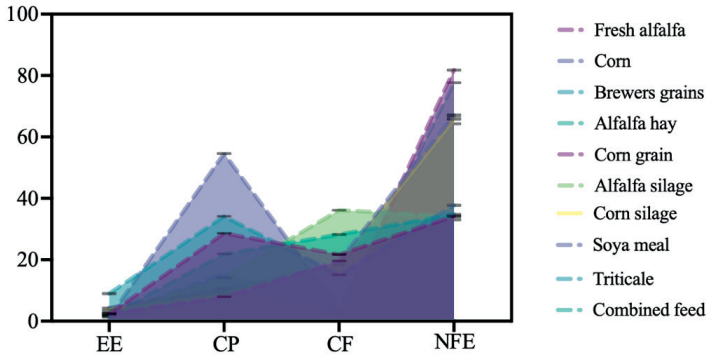


Figure 2. Organic matter (% DM) content in the analyzed feeds

The impact of feed chemical composition on pollution, particularly through greenhouse gas emissions, requires a detailed understanding of how the chemical components influence digestibility and methane production in the rumen; some of feed compounds directly affect the digestion rate and retention time within the digestive tract. More easily digestible feeds can help reduce greenhouse gas emissions, while feeds rich in insoluble fiber tend to increase the

volume of solid manure, complicating its management and heightening the risk of environmental pollution.

A key indicator in this context is the NES/CF ratio, indicating the fraction of fermentable carbohydrates in the diet. Feeds with increased NES/CF ratio allow easily metabolizable carbohydrates, stimulating propionic fermentation in the rumen, a reduction of producing methane in comparison to other fermentation types, helping to reduce methane emissions.

Based on the analyses carried out, most of the feeds in the evaluated ration exhibit a moderate NES/CF ratio. Feeds such as brewer's grains, green corn, corn silage, and combined feed show an optimal balance between NES and CF, making them highly digestible and associated with lower methane emissions than more fibrous feeds. These options not only contribute to reduced air pollution, but also have a lower ecological footprint, making them ideal for sustainable animal production.

Highly concentrated feeds, including corn, triticale, or soybean meal which contain NES have high digestibility and therefore low methane yield. However, their extensive use increase production costs and contribute to secondary forms of pollution, such as those associated with intensive agricultural practices (Matei et al., 2024b).

The assessed ration also contained low NES/CF ration feeds like alfalfa hay (1.23), green alfalfa (1.57) or alfalfa semi-silage (0.95). These fibrous feeds are generally less digestible and should be fed in limited quantities in order to reduce methane emissions. It is suggested to combine them with concentrated feeds to maintain dietary balance. Overall, less directly metabolizable energy sources (soybean meal or hay) for ruminal microbes are associated with anaerobic fermentation and thus with enhanced emissions of methane. In contrast, more easily digestible high-NES feeds, such as corn grain or corn silage, have the potential to limit potentially polluting discharges.

In particular, the protein content among the chemical constituents of feed contributes to soil pollution. High-protein diets carry the risk of higher environmental contamination, because excess proteins are wasted and excreted in the form of nitrogen, leading to soil and water contamination by nitrate leaching. Achieving an optimal balance between crude protein CP and non-nitrogenous extractives NES is essential to reduce this impact. Feeds rich in CP can result in higher ammonia emissions in urine and faeces, exacerbating nitrogen pollution, especially when protein intake exceeds the animal's nutritional needs. Analysis of the feeds included in the ration reveals important variations in CP content (Figure 3), which can directly affect nitrogen excretion levels and the environmental impact of animal production.

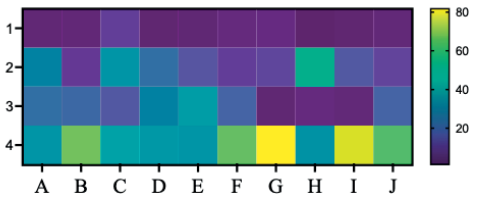


Figure 3. Variability in organic matter (% DM) contribution across the feeds included in the ration (1 - EE; 2 - CP; 3 - CF; 4 - NES; A - Fresh alfalfa; B - Corn; C - Brewers grains; D - Alfalfa hay; E - Corn grain; F - Alfalfa silage; G - Corn silage; H - Soya meal; I - Triticale; J - Combined feed)

Feeds such as green alfalfa, alfalfa hay, green corn, brewer's grains, corn silage, grain corn or triticale have a moderate protein content (1.25-13.33% DM), suggesting a low impact on nitrogen excretion. The high NES content in some of these forages such as green corn, corn silage, grain corn, triticale, contributes to maintaining an adequate nutritional balance, reducing the risk of exceeding the protein requirements of the animals. In contrast, soybean meal, with its high protein content (54.58% DM), can favor nitrogen excretion if not used efficiently, especially in animals with limited capacity to fully metabolize the proteins in their diet. However, this effect depends on the amount used in the ration, as well as in the main nutritional composition of the combined feed.

Previous studies (Ferket et al., 2002; Burkholder et al., 2007) have repeatedly emphasized the importance of efficient animal nutrition management to reduce the impact of environmental pollution. Carter & Kim (2013) pointed out the connection between animal feeding and environmental deterioration, associating suboptimal rations with higher rates of nitrogen and phosphorus discharge from manure. They concluded that excess nutrients provided in animals rations are not fully

assimilated for productivity, often contributing to nutrient pollution. These findings align with our observations, which indicate that the types of feed included in the analyzed ration have a considerable influence on nitrogen excretion level, especially in relation to their protein content and NES/CF ratio.

Some of feeds included in the analyzed ration presented a moderate NES/CF ratio, having a higher digestibility. These characteristics align with previous findings, which suggest that, adopting a balanced and well-formulated diet is sufficient to reduce nitrogen and phosphorus pollution. In contrast, feeds with a low NES/CF ratio, such as hays, are richer in insoluble fiber, which can lead to an increase in the volume of solid manure and thereby contribute to soil pollution. This effect is consistent with the studies of Hristov et al. (2006) who identified a direct link between excessive feed administration and soil pollution with nitrogen and phosphorus through their research on the quantitative control of potentially polluting nutrients in manure.

Chemical composition analysis of the studied ration showed that the protein content in the ration is also a remarkable contributor to soil pollution. High protein feeds like soybean meal can lead to nitrogen pollution as documented by studies carried out by Ferket et al. (2002); Knowlton et al. (2004) which demonstrated that excessive protein intake in the ration can lead to increased ammonia emissions. Furthermore, this protein surplus may disrupt nutritional balance of the ration, reducing the efficiency of nutrient conversion and causing important nutrient losses to the environment.

Regarding pollution reduction strategies, research has shown that the most effective practices involve optimizing rations to reduce nutrient waste. Sofia et al. (2020) pointed out that environmental pollution reduction must be accompanied by measures to increase the production efficiency and adopt ecological approaches. In this context, the integration of feeds with high digestibility and a balanced protein and cellulose content into rations can contribute to achieving these objectives. Also, as part of pollution reduction strategies, biotechnologies and the use of feed additives can be promising solutions to improve feed utilization and reduce the ecological impact of animal production.

CONCLUSIONS

The feed chemical composition influences both the efficiency of animal digestion and the environmental release of pollutants. Feeds high in crude fiber promote intense fermentation in the rumen, leading to increased emissions of methane and other greenhouse gases. Conversely, feeds rich in non-nitrogen extractives substances, like corn grain or corn silage, provide a quick source of energy, helping to reduce methane production. However, an unbalanced intake of crude protein, without proper alignment with NES levels can result in nitrogen losses through feces and urine, exacerbating pollution from nitrogen compounds. Achieving a balanced feed ration is essential to minimize the environmental impact, requiring the use of highly digestible feeds with an optimal ratio of crude fiber, crude protein, and non-nitrogenous extractives substances.

Reducing the environmental pollution caused by animal production through optimized animal nutrition and feed composition is achievable by targeted nutritional strategies that ensure the efficient use of feed resources.

These strategies include formulating balanced rations tailored to animal requirements, by such means reducing nitrogen and phosphorus excretions. These rations should incorporate highly digestible feeds, reducing waste and improving feed conversion efficiency into animal products. Additionally, the use of feed enzymes and other feed additives can enhance nutrient digestion and absorption of nutrients, while feed processing technologies like grinding and pelleting, improve nutrient availability and minimize lossed. To maximize effectiveness, these nutritional strategies should align with proper livestock management and optimized nutrient flows on the farm. These measures can be implemented as part of European and global initiatives aimed at pollution reduction, fostering more sustainable and efficient animal production to meet growing consumer demands.

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CANINE OBESITY: A CASE STUDY OF ROTTWEILERS AND THE IMPACT OF DIETARY MODIFICATION

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Abstract

Obesity is a disease that is increasingly recognised as a threat to pets, and organisations have been formed to raise awareness and prevent weight gain in dogs and cats. One of the breeds most affected by obesity is the Rottweiler, with studies confirming that obesity is the second most common disease in this breed. In the current study, a group of eight female Rottweilers from northern Romania were examined medically and nutritionally. The monitored females of the Rottweiler breed were divided into two categories according to the modification of the nutritional plan; therefore, for the first group of four females, both the type of food and the amount of food given were modified, and for the second group of females, the type of food was maintained, but the amount of food given daily was reduced. The study highlights that both reducing daily calorie intake and the use of specialized foods can be effective solutions for managing excess weight.

Key words*: dogs, female, food, nutrition, obesity, Rottweiler.*

INTRODUCTION

Obesity is a disease that is increasingly recognised as a threat to pets, and organisations have been formed to raise awareness and prevent weight gain in dogs and cats. Recent studies in the USA and Europe have shown alarmingly high rates of weight gain in dogs, with 59% of dogs in the USA (APOP, 2022) and between 6% and 69% of dogs in Europe being classified as overweight or obese by veterinarians or owners (Muñoz-Prieto, 2018).

One of the breeds most affected by obesity is the Rottweiler, with studies confirming that obesity is the second most common disease in this breed and that obesity in these dogs is associated with musculoskeletal diseases that are either exacerbated or caused by the degree of obesity in Rottweilers.

Obesity in dogs is a major health concern as it can contribute to a number of serious health issues including joint disease, cardiovascular disease and diabetes. It is also closely linked to certain breed characteristics, and Rottweilers are an important example in this context due to their robust build and tendency to gain weight quickly. In this regard, recent studies have emphasised the importance of careful dietary monitoring and weight management to prevent long-term complications.

MATERIALS AND METHODS

The monitored females of the Rottweiler breed were divided into two categories according to the modification of the nutritional plan; therefore, for the first group of four females, both the type of food and the amount of food given were modified, and for the second group of females, the type of food was maintained, but the amount of food given daily was reduced. The cases were managed both in a veterinary practice and in a kennel in north-eastern Romania, therefore the cases were recorded and followed up with the help of a veterinary software (Digitail) and by making individual files using Excel functions.

In order to observe the weight loss, the females were weighed monthly; during the first nutritional consultation the females were weighed, the height at the withers was recorded for each of them and the individual anamnetic data, food history, physical activity history and routine of each owner were recorded in order to observe possible changes that could be made to achieve a successful weight loss program.

RESULTS AND DISCUSSIONS

As research objectives, we aimed to present the effectiveness of two weight loss programs for Rottweiler females, one based on reducing the amount of food and the other based on the use of a type of food specifically designed for weight loss, also in the secondary aspect, the risks associated with obesity among Rottweiler females and its impact on general health were investigated and discussed.

Presentation of the groups: in the first group, Figure 2, the weights of the females that followed a weight management program with a type of feed adapted for the weight loss period were recorded, each female being given a code instead of her name, such as R1 to R4, because the owners' request not to publish the names was respected. In the second group, Figure 3, the weight variation was recorded for females R5, R6, R7 and R8 who followed the weight loss program that focused on decreasing the portion of food from the food they were already consuming at the time of the consultation.

The females included in the study were between 2 and 4 years of age, an age range that is specific to the young adult category according to the American Animal Hospital Association guidelines (AAHA, 2019). In terms of their reproductive status, they were intact and had not undergone ovariohysterectomy at the time of the nutritional consultation or during the study. They also had no history of gestation or parturition and were considered nulliparous at the time of the dietary assessment and monitoring.

Considering that the individuals analysed may have significant reproductive value, the owners decided to postpone their sterilisation. This decision was also influenced by uncertainties in the literature regarding the impact of sterilisation on longevity in the Rottweiler breed. Some recent studies, such as the one conducted by Joonè et al. (2023), suggest an increase in life expectancy in Rottweilers that have not undergone sterilisation. On the other hand, research focusing on multi-breed populations of dogs, such as that by Hoffman et al. (2018) or Urfer et al. (2019), supports the hypothesis that gonadectomy may contribute to an increase in life expectancy among dogs.

The eight females registered an average weight of 41.3 kg in January 2022, ranging from 37.3 to 46.5 kg, and a height at withers between 53 and 60 cm.

Only one of the eight females did not meet the height standard proposed by the AKC (2022), female R6 weighed 37.3 kg, and had a height at the withers of 53 cm, 3 cm less than the standard, and was classified in body score 5.5. Although the other females were within AKC standards, medical advice and owner experience considered weight loss beneficial to minimise possible orthopaedic risks from excess weight.

According to extensive research conducted on a population of 5321 Rottweilers from several veterinary clinics in England, it highlighted that among the most common conditions found in this breed were obesity and degenerative joint disease, with excess weight exacerbating osteoarticular symptoms, as highlighted in Table 1 (O'Neill, 2017).

Table 1 The occurrence of the most common diseases in Rottweilers (O'Neill, 2017)

Causes of death	Most commonly diagnosed diseases during life
Neoplasia	Aggressiveness (more common in males)
Inability to stand up	Obesity
Gastrointestinal disorders	Otitis
Neurocognitive disorders	Degenerative joint disorders
Undesirable behavior	Dental problems
Musculoskeletal difficulties	Pyotraumatic dermatitis

The nutritional plans were decided and implemented according to the individual anamnesis provided by the owners, and the results were tracked as follows: four females received the same food with only the amount of food decreased, and four females received a weight loss food with an energy density of 2668 kcal/kg.

By the time the nutritional weight-loss program was started, the females were fed a type of adult dry food designed for Rottweilers according to the manufacturer's specifications with an energy density of 3890 kcal/kg. The total amount of food offered daily being 550 g, fed in two portions, morning and evening, and weighed separately using a kitchen scale, each female received a total of 2140 kcal/day.

One essential aspect analysed during the nutritional consultation is the animal's feeding history. This includes details of the type(s) of food given, the actual amounts offered, and the number of daily meals.
In addition, information about the owner's daily schedule was requested to identify the pet's habits and the possibility of adjusting the number of meals per day.
Physical activity was also assessed, including the number of daily walks and the actual duration of play time.
Based on the level of activity, animals were categorised according to FEDIAF standards as follows:
- low activity: less than 60 minutes of physical activity per day, sedentary, inactive animal, spending most of the time sleeping or resting.
- moderate activity: between 60 minutes and 3 hours of daily physical activity.
- high activity: more than 3 hours of daily physical activity, alert, energetic and playful animal (FEDIAF, 2024).

In order to obtain the most realistic assessment, the questions about the animal's diet and activity level should be simplified so that the answers reflect the animal's daily routine as closely as possible (Petrescu et al., 2022).
The diet formulated for weight loss has a low energy density of 2.66 kcal/g, compared with 3.89 kcal/g for breed-specific diets (Figure 1). Reducing energy intake is a key element in body weight management, contributing to the caloric deficit required for weight loss (Petrescu et al., 2024).
As for the high fiber content of 17% in weight loss foods, studies claim that dietary fiber plays a key role in inducing satiety, which helps reduce spontaneous food intake. They also support optimal bowel transit, which is important for maintaining gastrointestinal health during a restrictive diet.
L-carnitine, also present in weight loss foods, is an essential nutrient involved in lipid metabolism. It facilitates the transport of fatty acids to mitochondria, where they are oxidized for energy production (Ringseis et al., 2013).

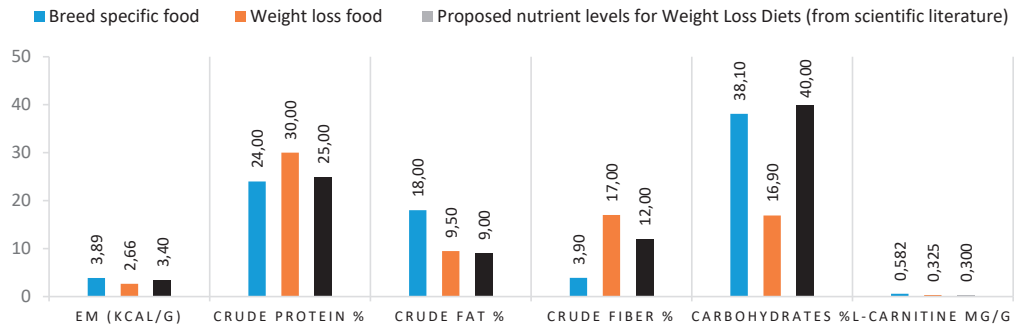
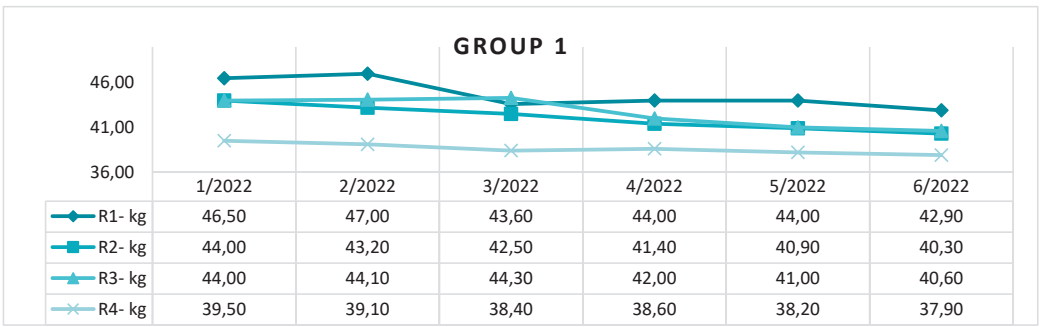
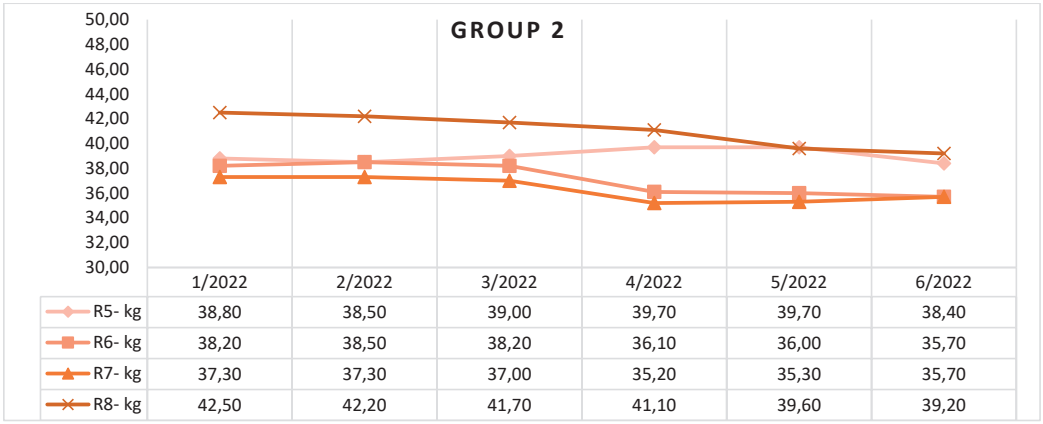


Figure 1. Comparison of nutrient composition and metabolizable energy of dog foods with recommended standards in the literature (Hand et al., 2010) - in black representation



	1/2022	2/2022	3/2022	4/2022	5/2022	6/2022
R1- kg	46,50	47,00	43,60	44,00	44,00	42,90
R2- kg	44,00	43,20	42,50	41,40	40,90	40,30
R3- kg	44,00	44,10	44,30	42,00	41,00	40,60
R4- kg	39,50	39,10	38,40	38,60	38,20	37,90

Figure 2. Group 1 - weight evolution of females (R1, R2, R3, R4) following a nutritional program with weight loss food



	1/2022	2/2022	3/2022	4/2022	5/2022	6/2022
R5- kg	38,80	38,50	39,00	39,70	39,70	38,40
R6- kg	38,20	38,50	38,20	36,10	36,00	35,70
R7- kg	37,30	37,30	37,00	35,20	35,30	35,70
R8- kg	42,50	42,20	41,70	41,10	39,60	39,20

Figure 3. Group 2 - weight evolution of females (R5, R6, R7, R8) following a nutritional program with breed specific food

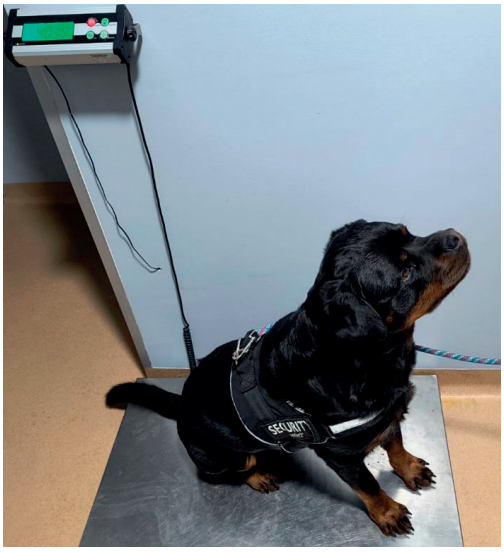


Figure 4. Physical appearance at the end of the nutritional program of the R2 female (own source)

In weight loss diets, L-carnitine helps to reduce fat mass by increasing the utilisation of fat as an energy source, and is particularly beneficial in overweight animals.

According to studies in the formulation of weight loss feeds an optimal protein intake should be ensured, observing that the weight loss feed shown in Figure 1, contains a crude protein intake of 30%, beneficial for the preservation of muscle mass during body weight reduction (German et al., 2010). At the same time, the carbohydrate level is low (16.9%) compared to breed specific feeds (33.1%), thus limiting the energy available from fast sources.



Figure 5. Physical aspect at the end of the nutritional program of the R2 female (own source)

This balancing supports the loss of body fat, while protecting muscle tissue.

There are several factors, however, that can limit the choice of these types of food for certain animals. These include human factors, such as the owners' desire to keep the same food that the animal previously consumed, citing behavioural reasons (e.g. picky eaters), fear of possible digestive problems that may arise from changing the diet, and financial considerations, as specialised weight loss food is often more expensive than standard adult dog food.

In the present study, it was chosen to maintain the same food, at the request of the owners of the females from group 2, in order to ensure consistency in the dog's diet and to facilitate a proper comparison between a food for adult dogs of the breed studied and a special low-fat, high-fiber diet intended for weight loss.

Table 2. Comparison of results obtained with weight loss food (group 1) and special breed food (group 2)

Name	Initial weight (kg)	Final weight (kg)	Weight loss duration (weeks)	Weight loss rate (%)	Average weight loss/week(%)	Initial energy intake (kcal)	DER calculated to achieve the proposed weight (kcal)
Group 1							
R1	46.5	42.9	21	7.7	0.37	2140	1154
R2	44	40.3	21	8.4	0.4	2140	1113
R3	44	40.6	21	7.7	0.37	2140	1113
R4	39.5	37.9	21	4	0.19	2140	1050
Group 2							
R5	38.8	38.4	21	1	0.05	2140	1050
R6	38.2	35.7	21	6.5	0.31	2140	1007
R7	37.3	35.7	21	4.3	0.2	2140	1007
R8	42.5	39.2	21	7.8	0.37	2140	1071

DER = daily energy requirement

The choice of the two types of feed allowed for a relevant comparative study, taking into account behavioral and animal comfort factors as well as financial and digestibility aspects.

A more efficient weight loss (Table 2; Figures 4 and 5) was observed with the use of the prescribed weight loss diet, with Group 1 females having an average weight loss rate of 6.95% compared to Group 2 females on a breed-specific weight management program. This difference is explained by the reduced fat content, controlled caloric intake, and optimised nutrient profile for reduced body mass, characteristic of the diet prescribed for obesity. In addition, all females were advised to increase physical activity by introducing longer walks and increased exercise compared to the previous level. Weight was monitored regularly, and the nutritional plan was adjusted according to individual progress.

CONCLUSIONS

In everyday practice, owner compliance with a new type of food is a significant challenge. Factors such as animal preferences, feed costs and owners' daily habits can influence the implementation of a feeding plan.

Not all cases of obesity can be managed solely by changing the type of food, as other pre-existing conditions such as metabolic disorders, urinary tract disorders, kidney diseases, etc. need to be taken into account. A personalised approach integrating both clinical and nutritional aspects is essential.

Regular weight monitoring and individualised adjustment of the nutritional plan have proven to be key factors in long-term success.

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RED PEPPER: NUTRITIONAL VALUE, CAROTENOIDS, ANTIOXIDANT CAPACITY, AND ITS USE IN BROILER DIET

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Abstract

Red pepper is a widely consumed vegetable, renowned for its impressive nutritional profile and significant health benefits, especially for its high content of vitamins and antioxidants. With a rich content of vitamin and essential carotenoid, red pepper plays a vital role in supporting immune function, improving iron absorption, and maintaining skin and eye health. The abundant carotenoid content of red peppers, which includes compounds such as capsanthin and capsorubin and gives the vegetables their characteristic red colour, significantly contributes to their antioxidant capacity. By effectively combating free radicals, these carotenoids lower the oxidative stress and inflammatory variables linked to the onset of chronic illnesses. Although there are many health benefits linked to consumption, there are still debates over their magnitude and the ways in which they work. Ongoing research continues to explore the bioavailability of carotenoids and their potential interactions with other dietary components, as well as the optimal ways to incorporate red pepper into various diets, both for its nutritional benefits and especially for improving the colour of meat in broiler.

Key words*: antioxidants, capsanthin, carotenoids, red pepper.*

INTRODUCTION

Various species of medicinal or aromatic plants, as well as spices, have been used for a long time, either for therapeutic or culinary purposes (Opara et al., 2014; Trifunschi et al., 2022). In recent years, consumers have grown increasingly interested in learning more about food fraud and authenticity as well as the consistency and safety of food products (Rațu et al., 2021). Research on natural preparations that are known to improve the quality of basic productions or have favourable effects on the health of animals has increased as a result of the prohibition on the use of antibiotics in animal husbandry due to the phenomena of antibiotic resistance (Steiner et al., 2014).

Given that 70-75% of production costs are related to feeding, the largest challenge in poultry farming is to ensure financial profitability while simultaneously providing adequate nutrition that is suited to each category of birds' physiological needs and performance potential (Usturoi et al., 2023). The most costly ingredients for compound feed are those that guarantee the required amount of protein (Ipek et al., 2009), components that vary greatly in terms of quality parameters, and most importantly, the purchase price, which is influenced by a variety of conjunctural and even geopolitical factors (Frempong et al., 2019). For instance, EU countries have production costs for one kilogramme of chicken meat that are about 45% higher than those of Brazil, one of the world's largest producers of soybeans (Magdelaine et al., 2003). Among the multitude of plants used in human and animal nutrition, the red pepper (*Capsicum annuum* L.) is of great interest as it is cultivated on very large areas worldwide. The pepper (hot pepper or chili) belongs to the Solanaceae family (which includes tomatoes, eggplants, potatoes, and tobacco), the *Capsicum* genus, which is composed of 27 different plant species (Zhuang et al., 2012).

Peppers can also be categorised as commercial products according to their intended use. For example, peppers from the species *Capsicum annuum* and *Capsicum frutescens* are grown for their strong spicy flavour and aroma, while red peppers are valued for their nutritional and therapeutic qualities as well as their high natural colour and antioxidant content (Krauss et al., 2000). Carotenoid pigments, which give fruits

their varied colours of yellow, orange, and red, are synthesised and accumulated by *Capsicum* species. The red colour of chilli pepper fruits is due to capsanthin, capsorubin, and capsanthin-5,6-epoxide, while the yellow-orange colour is mainly caused by the accumulation of α- and beta-carotene, zeaxanthin, lutein, and beta-cryptoxanthin (Reyes-Escogido et al., 2011). Pepper species have been used as experimental models for studying the biochemical and molecular aspects of carotenoid biosynthesis from various natural plant sources, despite the fact that the types and levels of carotenoids vary from one species to another and particularly depending on the environmental conditions in which they are grown (Gómez-García & Ochoa-Alejo, 2013).

MATERIALS AND METHODS

The evaluation of red pepper in terms of nutritional value, antioxidant capacity, and effects on broiler growth was based on the assessment of the state of knowledge from the PubMed and ResearchGate databases.

RED PEPPER: NUTRITIONAL VALUE

Due to their unique flavour, taste, and colour, as well as the fact that they contain a variety of minerals, bioactive substances, including antioxidants, fibre, and vitamin, red pepper and products made from it have a high nutritional value (Zhuang et al., 2012).

Red peppers are regarded one of the finest sources of natural colours. The essential components in red and green peppers are described in Table 1, and the nutritional characteristics of sweet peppers in Table 2. They have long been used as food colouring and more recently, as oleoresin since they are thought to be the vegetables with the highest concentrations of carotenoids. Carotenoid pigments, which are produced in large quantities during ripening, give capsicum its striking and distinctive red colour (Arimboor et al., 2015). With carbon atoms, nine conjugated double bonds in the core polyene chain, and several terminal groups that alter eche pigment's chromophore characteristics, all of the carotenoid pigments found in red pepper are isoprenoids. Red and yellow are the two isochrome families into which they can be divided. According to de Azevedo-Meleiro et al. (2009), the yellow fraction is composed of the remaining carotenoids, including zeaxanthin, beta-cryptoxanthin, and beta-carotene, while the red fraction primarily consists of capsanthin and capsorubin, which are thought to be unique to the *Capsicum* genus, and a smaller amount of other red carotenoids. The later fraction is regarded as the red one's biosynthetic precursor. Because of its simplicity and dependability, the ASTA method (American Spice Trade Association) is the most widely used analytical technique for determining the colour of paprika by extracting pigments using a selective solvent. The stability of carotenoids during heat drying is positively impacted by the presence of capsaicinoids in hot peppers (Gayathri et al., 2004).

Table 1. Main Nutritional Elements in Red and Green Bell Peppers

Element	Red pepper	Green pepper
K	3.27	3.55
Ca	1041	1009
P	1704	1528
Fe	73.6	64.7
Zn	18.6	25.0
Mn	22.5	21.3
S	1566	1518
Cu	7.94	11.6
Cl	2984	3250
Ca/P	0.61	0.66
Mn/Fe	0.306	0.329
Fe/Cu	9.27	5.58
Zn/Cu	2.34	2.16

The average values are expressed as a percentage on a dry weight basis.

Certain types of peppers provide twice as much vitamin C, antioxidant and nutrient, per gramme of fruit weight as tomatoes, apple, or orange (Wahyuni et al., 2013; Yehmed et al., 2023). Different pepper cultivars also contain various amounts of folic acid, a crucial B vitamin that lowers the risk of cancer and cardiovascular disorders (Phillips et al., 2006). The primary goal of pepper research is to identify the carotenoid makeup of the many different types. Carotenoid pigments, which are mostly produced during the ripening process, are responsible for the vibrant red hue of *Capsicum annum* fruits. The carotenoids responsible for the final red color of the fruits are capsanthin

and capsorubin (Minguez-Mosquera et al., 1994). The data obtained from the analysis conducted by researcher Ayuso et al., 2008 have been synthesized in Table 1 and Table 2, highlighting the most relevant nutritional characteristics of red pepper. These tables provide a detailed perspective on the chemical composition, energy value, and content of macro and micronutrients.

Table 2. Nutritional characterization of sweet peppers

Element	Red pepper	Green pepper
Fruit weight (g)	97.4	127
Moisture (%)	90.3	92.5
Protein (%)	11.9	11.7
Ash (%)	5.03	4.98
Fiber (%)	10.3	11.6
Firmness (N)	7.9	7.9
pH	5.2	6.0
Productivity (t/ha)	30.1	28.0

RED PEPPER: CAROTENOIDS

Carotenoids with over 50 different structures are responsible for the pepper's red colour (Guil-Guerrero et al., 2006; Giuffrida et al., 2013; Cervantes-Paz et al., 2014).

The unique keto carotenoids capsanthin, capsorubin, and cryptocapsin give ripe chilli pepper pods their vibrant red colour, while betacarotene, zeaxanthin, violaxanthin, and betacryptoxanthin give them their yellow-orange hue. While most of the xanthophylls in red peppers are found as esters and fatty acids, green pepper extracts are mainly composed of free carotenoids (Cervantes-Paz et al., 2014). In the majority of cultivars and variations, capsanthin accounts for 30-70% of the carotenoids. In the later phases of ripening, the ratios of capsanthin to capsorubin rise. Red pepper has also been found to contain significant amounts of neoxanthin, lutein, antheraxanthin, violaxanthin,capsanthin-5,6-epoxide, and other compounds (Hornero-Méndez et al., 2000).

Carotenoids are naturally occurring chemical molecules that can be yellow, orange, or red in colour and are found in plants, algae, and some microorganisms. Carotenoids and carotenoids (like beta-carotene and xanthophylls) are the two main categories of carotenoids (Hornero-Méndez & Mínguez-Mosquera, 1998). Eating foods high in carotenoid has been associated with several health benefits, such as protecting eye health and reducing oxidative stress. Numerous pharmacological benefits, including immunomodulatory, antibacterial, antidiabetic, neuroprotective, and anticancer capabilities, are provided by carotenoids to chickens (Bennedsen et al., 2000; Guerin et al., 2003; Arain et al., 2018). Dietary factors such as the kind and amount of carotenoid intake, foods containing carotenoids, and post-harvest processing, storage, and cooking techniques all affect the bioavailability of carotenoids (Guerin et al., 2003). Less than 10% of the 600 carotenoids found in natural sources have been found to be vitamin A precursors. Human tissues and blood contain a range of dietary carotenoids, both with and without provitamin A (Guerin et al., 2003, Pugliese et al., 2013). The most nutritionally active carotenoid is beta-carotene, which makes up 15-30% of the total carotenoids in serum. Carotenoids shield cells, tissues, and entire animals from a variety of neoplastic processes, enhance the immune system, inhibit mutagenesis, and lessen caused nuclear damage. Although carotenoids shield tissues from photons, some of them, such as carotene, have the ability to neutralise extremely reactive singlet oxygen and stop reactions caused by free radicals. Epidemiological studies have shown that eating fruits and vegetables high in carotenoids can protect against several cancers, especially lung cancer (Castañeda et al., 2005). Chilli pepper fruits synthesise and collect a variety of compounds, such as the unique capsaicinoids (hot chemicals), vitamins and colours (carotenoids) (Li et al., 2022). Several new carotenoids from paprika, such as mutatoxanthin, violaxanthin, antheraxanthin, lutein epoxide, and cryptocapsin, have also been reported. The keto groups in capsanthin and capsorubin are part of the conjugated double bond system (Castañeda et al., 2005).

Mature red chilli fruits may have a two to sixty-fold higher total carotenoid content than immature fruits. When black pepper fruits (*C. annuum*) ripen, for example, their total carotenoid concentration rises 66 times (Deli et al., 1992). During ripening, the carotenoid makeup also changes significantly. Generally speaking, lutein, beta-carotene, and neoxanthin are the most prevalent carotenoids in immature green chilli fruits; in green bell pepper, their respective percentages are lutein (40.8%), neoxanthin (15.1%), and beta-carotene (13.4%)

(Matsufuji et al., 2007). Both morphological and significant physiological changes occur in chili pepper fruits during ripening. These changes include alterations in pigment content and composition. These differences in fruit content are influenced by genetics, maturity, and growing conditions. Chilli pepper fruits are green because of chlorophyll, purple or violet because of anthocyanins, and yellow-orange because of beta-carotene, zeaxanthin, lutein, and beta-cryptoxanthin. Oxygenated carotenoids, such those with terminal acylcyclopentanol groups, control the red colour of chilli pepper fruits (Sun et al., 2007).

Beta-carotene (11.6%), capsanthin (35%), and violaxanthin (10%) are the three primary carotenoids found in red bell peppers. The carotenoids' makeup varies with fruit age (Curl et al., 1962). Other types of chilli peppers have also shown these changes in carotenoid makeup. Capsanthin, the main pigment, made about 46% of the Signal Red variety's total carotenoid content (Matsufuji et al., 2007). The total carotenoid content is also influenced by the variety. The fruits of *Asparagus officinalis*, Lilium, Aesculus, and Berberis, as well as the fruits of *Capsicum* red spp., are the primary sources of the red, lipophilic pigment known as capsanthin. During the ripening process, the amount of capsanthin in fruits can account for up to 50% of their total carotenoid content. Eleven conjugated double bonds, a conjugated keto group, and a cyclopentane ring make up capsanthin's structure. Because of these properties, capsanthin is a potent antioxidant (good radical scavenger). Despite having the same number of double bonds, capsanthin is more antioxidant than beta-carotene and beta-cryptoxanthin. Despite lacking the qualities of provitamin A, capsanthin is regarded as a beneficial molecule due to its antioxidant and antitumor (colon cancer) capabilities (Kim et al., 2009). According to Deruère et al. (1994), 95% of carotenoids gather in certain substructures called fibrils rather than being freely available or dispersed across chromoplasts. The conversion of preexisting photosynthetic pigments and de novo carotenoid production are the two metabolic processes that govern the amount and makeup of carotenoid profiles in chilli pepper fruits as they develop. For instance, research on the pigment content of Bola and Agridulce chilli pepper varieties at various ripening stages (green, colour break I, colour break II, red I, and red II) revealed that zeaxanthin, capsanthin, capsorubin, beta-cryptoxanthin, and capsorubin were synthesised from scratch, and that the concentrations of intermediates in the synthesis of red pigments (beta-carotene and violaxanthin) increased. In the meantime, in the red I and II phases (mature fruits), the chlorophyll pigments (lutein and neoxanthin) vanished (Minguez-Mosquera et al., 1994). However, violaxanthin accounted for 37% to 68% of the total carotenoids in the yellow-orange fruits of *C. baccatum, C. pubescens, and C. annuum.* Lutein (5% to 14%), cis-violaxanthin, and antheraxanthin were the next most important carotenoids (Rodríguez-Burruezo et al., 2010). In mature red chilli pepper fruits carotenoids have been observed to accumulate at the following rates, in descending order: zeaxanthin, or xanthophylls, neoxanthin, and violaxanthin), hydrocarbons (lycopene), ketocarotenoids (capsanthin, capsorubin, and capsolutein), and epoxixanthin-5,6-epoxide and capsanthin-3,6-epoxide. Due to their esterification with fatty acids, most carotenoids in ripe chilli pepper fruits are lipophilic (Minguez-Mosquera et al., 1994) and aid in their accumulation in lipophilic globules at the chromoplast level (Kim et al., 2004). The esterification process primarily occurs in de novo biosynthesised pigments such as capsanthin, capsorubin, zeaxanthin, and beta-cryptoxanthin, while it also occurs in previously synthesised pigments like violaxanthin. Biosynthesis and de novo esterification occur simultaneously during the ripening of chilli pepper fruits. While red xanthophylls, like capsanthin, are esterified by short-chain saturated fatty acids, such lauric acid, myristic, and palmitic acid, yellow xanthophylls are mostly esterified by myristic, palmitic , and a form of unsaturated linoleic acid. As a result, yellow xanthophylls have more double bonds in their fatty acid chains than their red counterparts, making them less stable (Mínguez-Mosquera et al., 1994). Similarly, free carotenoids and their partially and fully esterified forms make up 21.3%, 35.6%, and 43.1% of the total pigment content in fully ripe chilli pepper fruits (*Capsicum annuum* cv. Bola) (Mínguez-Mosquera et al., 1994). Furthermore,

it seems that the Numex, Mana, Belrubi, Negral, and Delfin types of fully ripe chilli pepper fruits (*C. annuum*) maintain a good balance between these three esterified fractions, which could serve as a "ripeness index". Remarkably, the process of carotenoid production does not depend on the breakdown of chlorophyll (Mínguez-Mosquera et al., 1994).

RED PEPPER: ANTIOXIDANT CAPACITY

The international food sector has a strong demand for the red pepper variety (*Capsicum annum* L.). Skins, stems, seeds, and leftover pulp are examples of by-products that are created after different phases of industrial processing and are thrown away as non-commercial goods (Rouhou et al., 2024). Peppers are also a rich source of oxygenated carotenoids (Márkus et al., 1999). Genetics and changes in maturity can affect their composition and concentration. Despite not having provitamin A activity, these substances are good at scavenging free radicals and may be crucial in avoiding cataracts and age-related macular degeneration (Seddon et al., 1994).

Epidemiological studies have demonstrated that antioxidants in fruits and vegetables can help prevent a variety of chronic diseases, such as cancer, heart disease, stroke, atherosclerosis, and cataracts (Trifunschi et al., 2022). In addition to their antioxidant function, carotenoids have other properties. For example, they exhibit anti-inflammatory and anti-tumor effects, improve cell communication, and aid in the detoxification of enzymatic systems (Khachik et al., 1999).

It is noted in the specialized literature that red pepper (RP) had the highest extraction yield, whereas green pepper (GP) had the lowest ($p > 0.001$). It is known that phenols and flavonoids have antioxidant effects on human health and nutrition (Otunola & Afolayan, 2013). Plant secondary metabolism is the source of phenolic chemicals and phenylpropanoids (Carvalho et al., 2019). The total polyphenol contents of GP and RP were 29.59 and 28.32 mg/g, respectively ($p > 0.001$). In line with the results of this study, Škrovánková et al. (2017) found that the total polyphenol content of paprika and peppers varied between 14.67 and 28.78 GAE mg/g. According to Carvalho et al. (2019), the ripening stage, variety, harvesting site, and year all affect the phenolic makeup of peppers. Ripening has an impact on the antioxidant properties of chilli pepper fruits as well. The antioxidant activity of chilli peppers is higher than that of green peppers. This disparity is easily explained by the increased levels of carotenoids, phenolics, flavonoids, and ascorbic acid present in fully ripe chilli pepper fruits (Sun et al., 2007). The levels of these antioxidants in chilli pepper fruits can vary depending on genotype, post-harvest management, and plant growth conditions (Deepa et al., 2007).

PERFORMANCE OF BROILERS FED RED CHILI PEPPER

According to studies (Galib et al., 2011; Shahverdi et al., 2013; EL-Gogary et al., 2024), chickens' weight gain, feed consumption, and conversion ratio all increased when red pepper was added to their diet (Table 3). Additionally, the use of red pepper decreased the levels of glucose, triglycerides, and cholesterol (p 0.05). The small intestine's mucosa and submucosa both significantly widened. According to this study, broiler given red pepper powder have larger small intestinal sections overall. It was determined that broiler performed better overall when red and black pepper were added to the feed at a rate of 1%. Both the overall quantity of bacteria and the number of coliform bacteria were significantly reduced when broiler were fed red pepper. Recent research indicates that adding 3% to the diet has a good impact on the production of broiler as well as the intestinal histology responses (El-Gogary et al., 2024).

Table 3. The amount of pepper administered to chicks based on their age and the subsequent performance

The age of the chicks (days)	Weight of administered peppers (grams/chick/day)	The performance of the chicks
0-7	0.1	Faster growth; Improved appetite; Stronger immunity.
8-14	0.25	Significant increase; Larger yolk; Increased resistance to diseases.
15-21	0.5	Accelerated growth; Reducing the risk of infections; Lower costs with medications

The age of the chicks (days)	Weight of administered peppers (grams/chick/day)	The performance of the chicks
22-30	0.75	Accelerated growth; The overall condition is better; Fewer respiratory problems.
30-42	1	Significant increase; The overall condition is better; Higher weight at slaughter; Fewer respiratory problems.

CONCLUSIONS

In accordance with the information available in the specialized literature, it can be stated that red pepper is a valuable source of carotenoids, making it an important natural agent for flavoring and coloring (the active compounds in the pepper fruit impart various shades for yellow, orange, and red colors).

Additionally, red bell peppers stand out for their remarkable nutritional value, due to their high content of vitamins (especially vitamin C) and essential minerals, which are important for the health of the immune system and skin.

In the same context, it should be noted that the high level of carotenoids (especially capsanthin and capsorubin) provides significant benefits for eye health and cell protection against oxidative stress, while also exerting an important antioxidant action, contributing to the reduction of risks associated with chronic diseases and premature aging.

Therefore, red pepper can be a valuable component in a balanced diet, having multiple positive effects on overall health.

Research on the stability of carotenoids extracted from red peppers in food products has been conducted according to appropriate protocols and working methods, and the results obtained are essential for evaluating their potential as food coloring agents; additionally, technological progress has led to the development of new methodologies and processes that help reduce issues of oxidative, acidic, and solubility instability of carotenoids.

The conclusion of this study is that supplementing the feed of broiler with preparations based on red pepper can induce positive effects at the productive level (increased growth rate, reduced feed consumption, and improved overall health), as well as on the quality of the final product (intensification of the yellow color of the meat and skin).

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MEALWORM (*Tenebrio molitor*) AS A PROTEIN SOURCE: EFFECTS ON GROWTH PERFORMANCE, CARCASS TRAITS AND NITROGEN EXCRETION IN QUAILS

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Abstract

This study aimed to assess the impact of substituting soybean meal with mealworm (Tenebrio molitor L.) as the primary protein source in compound feeds on growth performance, carcass traits, nutrient digestibility and nitrogen excretion in Japanese quails. A total of 120 one-day-old quails were assigned to four groups, each with 10 replications, and the experiment lasted 35 days. The control group was fed a 34% soybean meal-based diet, while the experimental groups had 1.4% soybean meal replaced with 2.8% or 5.6% mealworm meal. Lowest body weight gain and feed consumption occurred in the 1.4% mealworm group (P<0.05), with mealworm content displaying a cubic effect. The 5.6% mealworm group exhibited significantly improved feed efficiency (P<0.05). Furthermore, mealworm supplementation significantly increased the digestibility of dry matter, crude ash, and metabolizable energy (P<0.001). Moreover, fecal nitrogen excretion and nitrogen retention rates increased linearly with mealworm supplementation (P<0.001). In conclusion, Mealworm can replace 5.6% soybean meal in quail diets, improving growth and protein use as a sustainable alternative.

Key words*: broiler quails, digestibility, growth performance, mealworm (Tenebrio molitor L.), nitrogen excretion.*

INTRODUCTION

The search for sustainable and alternative protein sources in animal feed has become a critical focus in modern livestock and poultry production due to increasing concerns about feed costs, environmental sustainability, and the limitations of conventional protein sources such as soybean meal and fishmeal (van Huis et al., 2013; Oonincx et al., 2015; Gasco et al., 2019). In this context, insect-based proteins have emerged as a promising alternative due to their high nutritional value, rapid growth, and ability to be produced with minimal environmental impact (Mekonnen & Hoekstra, 2012; Smetana et al., 2015). Among the various insect species evaluated for feed applications, mealworms (*Tenebrio molitor*) have gained particular attention due to their relatively high protein and fat content, as well as their efficient conversion of organic waste into valuable nutrients (Dobermann et al., 2017; Halloran et al., 2016; Ramos-Elorduy et al., 2017).

The incorporation of mealworm meal into poultry diets has been explored for its potential to enhance growth performance, improve feed efficiency, and contribute to environmental sustainability by reducing nitrogen excretion (Almeida et al., 2019; Khan et al., 2021). Quail (*Coturnix coturnix japonica*) is an economically significant species in the poultry industry, known for its rapid growth, early sexual maturity, and high reproductive efficiency, making it an ideal model for research on alternative feed ingredients (Hassan et al., 2021; Cullere & Dabbou, 2021). However, studies on the effects of mealworm inclusion in quail diets remain limited, particularly concerning growth performance, feed efficiency, carcass traits, and nitrogen metabolism - critical factors for assessing the sustainability and environmental impact of animal production systems (Iji et al., 2017).

The nutritional composition of mealworms includes high-quality protein, essential amino acids, and unsaturated fatty acids, which suggest potential benefits for poultry growth and development (Liu et al., 2013; Van der Borght et al., 2022). Additionally, the inclusion of insect meal in poultry diets may influence carcass traits by affecting body composition, muscle growth, and fat deposition (van der Fels-Klerx et al., 2018; Bovera et al., 2016). Nutrient digestibility and nitrogen excretion are also essential considerations when evaluating the ecological footprint of poultry production, as

excessive nitrogen output contributes to environmental pollution through ammonia emissions and nitrate leaching (Mottet et al., 2017; Wang et al., 2020). The digestibility of nutrients, particularly proteins, and the impact on nitrogen excretion are also vital considerations in assessing the ecological footprint of poultry production. Reduced nitrogen excretion is an important factor in minimizing the environmental impact of livestock farming, as nitrogen compounds, such as ammonia and nitrate, can contribute to water and air pollution (Mottet et al., 2017).

This study aims to investigate the effects of mealworm inclusion in quail diets on growth performance, carcass characteristics, nutrient digestibility, and nitrogen excretion. By integrating mealworms as a protein source in quail feed, this research seeks to provide insights into their viability as a sustainable alternative protein source, supporting both economic and environmental benefits in poultry production.

MATERIALS AND METHODS

This study was conducted following ethical guidelines and was approved by the Dicle University Experimental Animal Ethics Committee (DÜHADEK) under protocol number 2022/201. A total of 120 one-day-old Japanese broiler quails (*Coturnix coturnix japonica*) were randomly assigned to four dietary treatment groups, each consisting of 30 birds. Each treatment group was further divided into 10 replicates, with three birds per replicate. The experiment lasted for 35 days

The control group received a basal diet containing 34% soybean meal as the primary protein source. In the experimental groups, soybean meal was partially replaced with mealworm (*Tenebrio molitor* L.) meal at inclusion levels of 1.4%, 2.8%, and 5.6%. All diets were formulated to meet the nutrient requirements of Japanese quails as per the National Research Council (NRC, 1994). The mealworms were obtained from a commercial supplier and processed by drying at 60°C for 24 hours before being ground into a fine powder for feed formulation.

The quails were housed in a controlled environment within a cage-based broiler quail test coop at the Department of Animal Science, Faculty of Agriculture, Dicle University. The room temperature was maintained at 25°C with 60% relative humidity. A 23-hour light and 1-hour dark cycle was applied throughout the experiment. Quails were provided with *ad libitum* access to feed and water.

The dietary treatments were formulated to ensure similar protein levels among the groups, with mealworm meal being used to replace part of the soybean meal. The ingredients and composition of the experiment dies is shown in Table 1.

Table. 1 Ingredients, composition and nutrient content levels of quail's diets

Ingredients	%			
	MW0	MW1.4	MW2.8	MW5.6
Wheat	5.00	5.00	5.00	5.00
Maize	57.00	57.00	57.00	57.00
Soybean Meal (48% CP)	34.00	32.60	31.20	28.40
Mealworm Meal	0.00	1.40	2.80	5.60
Dicalcium Phosphate[a]	1.00	1.00	1.00	1.00
Bone Meal (30% Ca, 13% CP)	2.00	2.00	2.00	2.00
DL-Methionine	0.15	0.15	0.15	0.15
L-Lysine	0.20	0.20	0.20	0.20
NaCl	0.40	0.40	0.40	0.40
Vitamin and Mineral Premix[b]	0.25	0.25	0.25	0.25
Parameters	**Analysis values, %**			
Dry Matter	89.7	89.7	89.7	89.7
Crude Protein	22.1	22.1	22.1	22.1
Ether extract	2.12	2.12	2.12	2.12
Crude Ash	5.90	5.90	5.90	5.90
Crude Fiber	2.73	2.73	2.73	2.73
Parameters	**Calculated values, %**			
Metabolizable energy (ME poultry), kcal/kg	2910	2910	2910	2910
Ca	0.96	0.96	0.96	0.96
Available Phosphorus	0.60	0.60	0.60	0.60
Na	0.19	0.19	0.19	0.19
Cl	0.28	0.28	0.28	0.28
Methionine + Cysteine	0.88	0.88	0.88	0.88
Lysine	1.33	1.33	1.33	1.33
Threonine	0.82	0.82	0.82	0.82
Tryptophan	0.29	0.29	0.29	0.29
Linoleic acid	1.20	1.20	1.20	1.20
Electrolyte Balance (mEq/kg DM)	251	251	251	251

[a]Composition: 240 g Ca and 17.5 g P/kg;
[b]Composition (per kg feed): Vitamin A, 8,000 IU; Vitamin D3, 1,200 IU; Vitamin E, 10 IU; Vitamin K3, 2 mg; Thiamine, 2 mg; Riboflavin, 5 mg; Pyridoxine, 0.2 mg; Vitamin B12, 0.03 mg; Pantothenic acid, 10 mg; Niacin, 50 mg; Biotin, 0.1 mg; Folic acid, 0.5 mg; Iron, 80 mg; Zinc, 40 mg; Manganese, 60 mg; Iodine, 0.8 mg; Copper, 8 mg; Selenium, 0.2 mg; Cobalt, 0.4 mg.
[c]Composition (per kg feed): Fe, 80 mg; Zn, 40 mg; Mn, 60 mg; I, 0.8 mg; Cu, 8 mg; Se, 0.2 mg; Co, 0.4 mg.

The mealworms used in the experiment were purchased from a commercial supplier, and nutrient analysis was conducted. The nutrient contents are shown in Table 2.

Table 2. Nutrient content of mealworm (%)

Parameters	%
Dry Matter	89.5
Crude Protein	56.1
Crude Fat	25.5
Crude Fiber	5.5
Crude Ash	2.4

The diets were balanced to provide similar protein and energy content. The proximate composition of the experimental diets was determined by standard methods (AOAC, 2005), and the nutrient content of each diet (crude protein, crude fat, ash, fibre, calcium, and phosphorus) was calculated to meet the requirements for broiler quails.

Growth performance was evaluated by measuring body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Body weights were recorded weekly, and feed intake was measured by subtracting the amount of feed remaining from the total amount of feed provided. FCR was calculated as the ratio of feed intake to body weight gain. These parameters were measured for all birds throughout the 35-day experimental period.

Nutrient digestibility was assessed during the final week of the experiment using the total collection method (Schneider & Flatt, 1975). A total of 12 birds from each treatment group (3 birds per replicate) were selected and housed individually in metabolic cages. The quails were acclimatized to the cages for 7 days before the collection period, which lasted 5 days. Faeces were collected daily and stored at -20°C until analysis. The digestibility coefficients for dry matter (DM), crude ash (CA), and metabolizable energy (ME) were calculated based on the difference between the nutrients provided and those excreted in the faeces.

Nitrogen excretion was determined during the same period as the digestibility trial. Nitrogen intake was calculated based on the nitrogen content of the diets, and nitrogen excretion was determined by analyzing the nitrogen content of the faeces. The nitrogen balance was calculated as the difference between nitrogen intake and nitrogen excretion. The results were expressed as fecal nitrogen excretion and nitrogen retention rates per bird per day. Nitrogen excretion and retention rates were analyzed for each treatment group.

At the end of the experiment, six quails per treatment were randomly selected, fasted for 12 hours, and slaughtered by cervical dislocation. Carcass weight, carcass yield, gizzard weight, and intestinal weight were recorded and expressed as a percentage of live body weight.

Data were analyzed using one-way analysis of variance (ANOVA) in SPSS software (version 18.0, IBM Corp., Armonk, NY, USA). Differences between treatment means were evaluated using Tukey's multiple comparison test at a significance level of $P<0.05$. Regression analysis was applied to assess linear, quadratic, and cubic trends in response to mealworm inclusion levels.

RESULTS AND DISCUSSIONS

The effects of replacing soybean meal with mealworms in the diet on body weight gain feed intake and feed conversion ratio of broiler quails are detailed in Table 3. The observation that quails fed with 5.6% mealworm meal achieved the highest body weight gain is in line with studies by Schiavone et al. (2017) and Liu et al. (2018), which found that the inclusion of insect meals, such as mealworms, in poultry diets led to enhanced growth performance. For instance, Schiavone et al. (2017) observed significant improvements in the growth of broilers fed diets supplemented with insect meal, which provided high-quality protein. This is likely due to the balanced amino acid profile and the higher digestibility of insect proteins, which support better muscle development and growth. Similarly, Liu et al. (2018) reported that higher levels of mealworm meal led to increased body weight gain in broilers, supporting the notion that mealworm meal can contribute to improved growth in poultry.

The reduction in feed intake observed as mealworm meal inclusion increased is consistent with findings from Folk et al. (2019), who noted a decrease in feed consumption as insect-based meals were included in broiler diets. This decrease could be attributed to several factors, including the higher nutrient density of mealworm meal, which could lead to

satiation at lower feed volumes. Additionally, changes in the palatability of the diet due to the inclusion of insect meal could affect intake levels, as suggested by Belluco et al. (2013). The study by Kidd et al. (2018) also suggests that the inclusion of insect meals could alter the feed's sensory properties, potentially leading to reduced consumption if the feed is not well-accepted by the birds.

The significant improvement in feed conversion rate at the 5.6% mealworm meal level is consistent with results from Oliviero et al. (2017), who found that broilers fed diets containing insect-based protein sources, such as mealworms, had improved feed conversion ratios compared to those fed conventional protein sources. The increased feed conversion rate in the current study suggests that mealworm meal, at higher inclusion rates, allows quails to utilize feed more effectively. This could be due to the high-fat content of mealworm meal, which provides a concentrated energy source, as well as the optimal amino acid profile that supports better protein synthesis and muscle growth. However, it is important to note that the optimal inclusion level of mealworm meal varies across species and production conditions. For example, Ramos-Elorduy et al. (2014) found that the inclusion of mealworm meal at 10% in poultry diets resulted in improved growth performance in chickens, but higher inclusion rates could reduce feed intake, as was observed in our study. This suggests that while mealworm meal is a promising alternative protein source, its inclusion should be carefully balanced to optimize both feed intake and feed conversion rate. Our results support Morsy's (2022) observation that 10% mealworm inclusion enhances growth performance, while contrasting with Secci et al. (2022), who reported detrimental effects beyond 3.3% inclusion. Additionally, it is worth considering that mealworm meal's nutrient profile, including its high levels of lipids and proteins, can contribute to more efficient growth when included in poultry diets. Ravi et al. (2020) highlighted that insect meals, including those from mealworms, provide a balanced nutrient composition that supports better feed conversion and growth, which is evident in our findings where the highest level of mealworm meal (5.6%) improved feed efficiency.

The effects of replacing soybean meal with mealworms in the diet of quails on carcass weight, yield, and slaughter parameters were shown in Table 4. The findings indicated that there was no statistically significant difference in carcass weight between the experimental groups ($P>0.05$). Similarly, carcass yield values did not show any significant variation among the groups ($P>0.05$). When examining the findings related to the intestinal weight ratio within slaughter parameters, the differences between the groups were also found to be insignificant ($P>0.05$). Likewise, the gizzard weight ratio did not show statistically significant differences among the experimental groups ($P>0.05$). Throughout the experiment, mealworm supplementation at levels of 1.4%, 2.8%, and 5.6% in compound feed did not affect carcass weight, yield, or slaughter parameters. These findings are consistent with the study conducted by Sedgh-Gooya et al. (2021), in which mealworms were added at 2.5% and 5% as a replacement for corn-soybean meal in broiler feed. Additionally, similar findings were reported by Sabırlı & Çufadar (2019), Elahi et al. (2020), and Ait-Kaki et al. (2021), further supporting the conclusions of the present study. Overall, this research aligns with previous literature, demonstrating that mealworm supplementation at the tested levels does not significantly alter carcass and slaughter parameters.

Nutrient digestibility and nitrogen excretion are presented in Table 5 and Table 6. In this study, the presence of mealworms (*Tenebrio molitor* L.) in the diet was found to increase dry matter digestibility linearly ($P<0.05$) but did not create a cubic or quadratic effect ($P>0.05$). Compound feeds containing different levels of mealworms had similar effects on dry matter digestibility. However, diets containing 2.8% and 5.6% mealworms significantly improved dry matter digestibility compared to the control group ($P<0.05$). No statistically significant difference was observed in crude fiber (CF) digestion between the control and experimental groups ($P>0.05$). In terms of metabolizable energy (AME) utilization, the difference between the control group and the 1.4% mealworm group was insignificant ($P>0.05$), but the 2.8% and 5.6% mealworm groups exhibited significantly higher values than the other groups ($P<0.05$).

Crude ash (CA) digestibility was significantly higher in the control group compared to the experimental groups (P<0.05), while no significant difference was found among the other experimental groups. Regarding calcium (Ca) and phosphorus (P) digestibility, no significant differences were detected between the research groups (P>0.05). While crude fiber, Ca, and P digestibility did not show significant variations, dry matter and apparent metabolizable energy (AME) parameters increased proportionally with the level of mealworms in the compound feed. Cullere et al. (2016) conducted a study on broiler quail diets, replacing soybean meal with 0%, 10%, and 15% black soldier fly (*Hermetia illucens*) larvae meal, and reported that increasing the larvae meal proportion positively influenced apparent metabolizable energy values. This finding aligns with the results of our study. However, Kanoğlu (2019) found no significant difference in energy efficiency between a group receiving 0% mealworms and a group in which fish meal was completely replaced by mealworms in broiler quail diets. This result contradicts our study, and the discrepancy is thought to be due to the use of defatted mealworms in Kanoğlu's study. In our study, where mealworms were used as a soybean meal replacement, an increase in the proportion of mealworms in compound feeds enhanced dry matter digestibility. Similarly, Jin et al. (2016) found that the proportional increase of dried mealworms in the diet of weaned piglets improved dry matter digestibility. However, Parlar (2023), in a study on broiler chicken diets replacing fish meal with 25%, 50%, 75%, and 100% mealworms, reported that an increased proportion of mealworms negatively affected dry matter digestibility. This result contrasts with our findings, possibly due to the substitution of fish meal with mealworms in that study. The higher digestibility of fish meal compared to mealworms may explain this discrepancy. No statistically significant difference was found in crude protein intake among the research groups (P>0.05). Fecal crude protein excretion in the experimental groups did not differ among themselves but was significantly lower than in the control group (P<0.05). Similarly, Oonincx et al. (2010) stated that mealworms did not increase ammonia emissions when used in human and animal nutrition. Among the experimental groups, the highest crude protein retention rate was observed in the 5.6% mealworm group, followed by the control group. No statistically significant differences were found among the other groups (P>0.05). Crude protein digestibility was significantly higher in the 2.8% and 5.6% mealworm groups compared to the control and 1.4% mealworm groups (P<0.05), while differences between the control and 1.4% groups were insignificant (P>0.05). Our results indicated that adding 1.4%, 2.8%, and 5.6% mealworms to Japanese quail diets improved digestible crude protein, reduced nitrogen excretion, and enhanced nitrogen retention. Similarly, Jin et al. (2016) found that increasing dried mealworm levels in weaned pig diets resulted in a linear increase in crude protein, dry matter, nitrogen retention, and digestibility, which aligns with our findings. However, Bovera et al. (2015) reported that adding mealworms to broiler diets reduced protein utilization but improved the European efficiency factor, partially aligning with our study. Conversely, Cullere et al. (2016) found that increasing black soldier fly (*Hermetia illucens*) larvae meal levels in quail diets reduced digestible crude protein, contradicting our study's results. Differences in the type of insect used, feed formulation, and experimental conditions could explain this disparity.

Table 3. Effects of replacing soybean meal with mealworms in the diet on the growth performance of broiler quails

Measurements	Mealworm Meal Levels, %				SEM	P	Effects[1]		
	0 (Control)	1.4	2.8	5.6			L	Q	C
Body Weight Gain, g/quail	189.8^{ab}	177.5^{b}	192.1^{ab}	204.1^{a}	2.268	0.040	NS[3]	*	*
Feed Intake, g/quail	750.6^{a}	659.8^{b}	712.5^{ab}	724.9^{ab}	10.627	0.014	NS	*	**
Feed Conversion rate (FI/BWG)	4.01^{a}	3.72^{ab}	3.72^{ab}	3.51^{b}	0.071	0.053	*	NS	NS
Mortality, %	4.01^{a}	3.72^{ab}	3.72^{ab}	3.51^{b}	0.071	0.053	*	NS	NS

Mean values with different superscripts $^{(a,b)}$ within the same row are statistically different at *P < 0.05 and/or **P < 0.01.
[1]Effects: L = Linear, Q = quadratic, C = cubic, [2]SEM = pooled standard error of the mean, [3]NS = not significant.

Table 4. Effects of replacing soybean meal with mealworms in the diet on carcass traits and internal organ weight of broiler quails

Measurements	Mealworm Meal Levels, %				SEM	P	Effects[1]		
	0 (Control)	1.4	2.8	5.6			L	Q	C
Carcass Weight, g	134.0	131.7	131.9	137.1	1.923	0.742	NS	NS	NS
Carcass Yield, %	68.8	68.3	68.3	68.6	0.371	0.946	NS	NS	NS
Intestine Weight Ratio (g/g BW)	3.2	3.0	2.9	3.1	0.058	0.429	NS	NS	NS
Gizzard Weight Ratio (g/g BW)	2.01	2.05	2.12	2.13	0.036	0.866	NS	NS	NS

Mean values with different superscripts [(a,b)] within the same row are statistically different at *P < 0.05 and/or **P < 0.01.
[1]Effects: L = Linear, Q = quadratic, C = cubic, [2]SEM = pooled standard error of the mean, [3]NS = not significant.

Table 5. Effects of replacing soybean meal with mealworms in the diet on nutrient digestibility of broiler quails

Measurements	Mealworm Meal Levels, %				SEM	P	Effects[1]		
	0 (Control)	1.4	2.8	5.6			L	Q	C
Dry Matter, %	74.0^{b}	74.7^{ab}	76.4^{a}	76.8^{a}	0.34	0.011	**	NS	NS
Crude Fiber, %	43.7	42.5	44.0	43.6	0.41	0.428	NS	NS	NS
AME, kcal/kg	6.19^{b}	6.35^{b}	7.10^{a}	7.32^{a}	0.13	0.001	**	NS	NS
Crude Ash, %	41.4^{a}	42.6^{b}	42,0^{b}	42,2^{b}	0.37	0.04	*	*	NS
Ca	51.2	50.8	52.4	52.0.	0,44	0.128	NS	NS	NS
P	45.6	45.0	46.1	46.2	0.38	0.227	NS	NS	NS

Mean values with different superscripts [(a,b)] within the same row are statistically different at *P < 0.05 and/or **P < 0.01.
[1]Effects: L = Linear, Q = quadratic, C = cubic, [2]SEM = pooled standard error of the mean, [3]NS = not significant.

Table 6. Effects of Replacing Soybean Meal with Mealworms in the Diet on Nitrogen Digestibility of Broiler Quails

Measurements	Mealworm Meal Levels, %				SEM	P	Effects[1]		
	0 (Control)	1.4	2.8	5.6			L	Q	C
Nitrogen intake (g/quail/day)	6.12	6.03	6.14	6.20	0.04	0.607	NS	NS	NS
Fecal nitrogen excretion (g/quail/day)	2.49^{a}	2.30^{b}	2.23^{b}	2.14^{b}	0.03^{b}	0.001	**	NS	NS
Nitrogen retention (g/guail/day)	3.63^{b}	3.73^{ab}	3.92^{ab}	4.07^{a}	0.06	0.003	**	NS	NS
Crude protein digestibility (%)	59.4^{b}	61.8^{ab}	63.7^{a}	65.6^{a}	3.11	0.045	**	NS	NS

Mean values with different superscripts [(a,b)] within the same row are statistically different at *P < 0.05 and/or **P < 0.01.
[1]Effects: L = Linear, Q = quadratic, C = cubic, [2]SEM = pooled standard error of the mean, [3]NS = not significant.

CONCLUSIONS

The inclusion of 5.6% mealworms resulted in the highest body weight gain and feed efficiency while reducing nitrogen excretion, contributing to more environmentally friendly production. Mealworms increased the digestibility of dry matter, crude ash, and metabolizable energy but had no significant effect on Ca and P digestibility. In conclusion, mealworms can be considered a sustainable and alternative protein source in animal feed.

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EFFECTS OF BORIC ACID AND BORAX PENTAHYDRATE ON PERFORMANCE, EGG QUALITY TRAITS AND BONE MINERALIZATION IN LAYING HENS

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Abstract

This study evaluated the effects of dietary supplementation with boric acid and borax pentahydrate at two levels (50 and 100 mg/kg) on production performance, egg quality traits, and bone mineralization in laying hens. A total of 315 Atak-S hens were randomly assigned to five groups: a control group and four treatment groups. The inclusion of 100 mg/kg borax pentahydrate in the diet significantly improved egg production, reduced feed intake, and resulted in the most efficient feed conversion ratio ($P < 0.05$). Moreover, boron supplementation significantly increased eggshell thickness, particularly in the borax pentahydrate group ($P < 0.05$), while internal egg quality parameters (Haugh unit and albumen index) remained unaffected ($P > 0.05$). A slight reduction in yolk redness was observed at higher boron levels. However, dietary boron supplementation had no significant effect on bone mineralization parameters ($P > 0.05$). Overall, the findings suggest that dietary boron, especially in the form of borax pentahydrate, can enhance productivity and eggshell quality in laying hens without negatively affecting internal egg characteristics.

Key words*: boric acid, borax pentahydrate, egg quality traits, laying hens, productive performance.*

INTRODUCTION

Boron, an essential trace element, plays a significant role in various physiological processes in animals, including bone development, mineral metabolism, and enzymatic functions (Hunt, 1994). In recent years, boron has gained attention in animal nutrition, particularly in poultry production, for its potential benefits, despite not being classified as an essential nutrient (Nielsen, 2014). Research has highlighted its importance in improving growth performance, eggshell quality, and overall mineralization in laying hens (Rossi et al., 2018). Boron supplementation has been associated with enhanced reproductive health, bone strength, and production efficiency in poultry, with studies suggesting its positive effects on calcium metabolism and the utilization of other minerals like magnesium and phosphorus (Kucuk et al., 2003; Wilson & Ruszler, 1997; Hunt, 1994).

The effectiveness of boron supplementation in animal diets largely depends on its form and concentration. Boric acid and borax pentahydrate are two commonly used boron sources, each with distinct chemical properties and bioavailability (Devirian & Volpe, 2003). Research has demonstrated that dietary boron supplementation can enhance egg production and shell quality in laying hens, but the optimal form and dosage remain unclear (Eren et al., 2004). While studies have primarily focused on boron's effects on eggshell quality and bone mineralization, there is limited information on its impact on internal egg quality parameters, such as albumen and yolk characteristics. Recent findings suggest that boron supplementation in poultry diets may improve egg production, egg quality, and overall health (Eren et al., 2004). For example, Nielsen (2000) highlighted boron's role in enhancing calcium metabolism and bone strength in mammals, suggesting similar benefits for poultry. Eren et al. (2012) further demonstrated that dietary boron positively influences eggshell quality and mechanical properties in laying hens. Additionally, Bozkurt et al. (2015) found that boron supplementation improved eggshell thickness and strength, emphasizing its role in mineral utilization. Similarly, Khattak et al. (2018) reported that dietary boron enhanced bone mineralization and tibia

strength in broilers, indicating its potential to support skeletal integrity in laying hens. These findings underscore the need for further research to optimize boron supplementation strategies for poultry production. Various boron compounds, including boric acid, borax, and sodium tetraborate, have been evaluated in poultry diets. For instance, Kabu and Akosman (2013) found that boric acid supplementation at different levels positively influenced calcium and phosphorus metabolism, contributing to improved eggshell formation. Kurtoğlu et al. (2005) further indicated that dietary boron supplementation, regardless of the source, significantly improved bone mineralization and reduced osteoporosis in aged hens. Beyond its effects on bone and eggshell health, boron has been shown to influence immune response and antioxidant enzyme activity. Pradhan et al. (2021) reported that boron supplementation improved immune function by increasing antioxidant capacity and reducing oxidative stress in poultry. Oğuz et al. (2017) also found that dietary boron enhanced the production of immune-related cytokines, strengthening disease resistance. In terms of feed efficiency, Akhavan-Salamat and Ghasemi (2020) explored boron's effects on nutrient absorption and gut health, highlighting its potential to improve feed conversion ratios. Mohammadpour et al. (2016) supported these findings, showing that boron supplementation improved intestinal morphology, nutrient digestibility, and overall performance in broilers. However, there is limited research on the specific effects of boric acid and borax pentahydrate supplementation in laying hens, particularly regarding their impact on yield performance, egg traits, and mineral deposition, underscoring the need for further investigation in this area. Understanding the effects of these boron sources on laying hens is essential for optimizing dietary formulations and enhancing egg production performance. This study aims to evaluate the impact of boric acid and borax pentahydrate on yield performance, egg traits, and mineralization in laying hens. By exploring these parameters, the research will contribute to the broader field of poultry nutrition and provide practical insights for the poultry industry.

MATERIALS AND METHODS

This research was carried out in compliance with ethical standards and received approval from the Dicle University Experimental Animal Ethics Committee (DÜHADEK) under protocol number 2022/202.

Atak-S hens, at 23 weeks of age, were housed in enriched cage systems (n = 315; 45 cages; 7 hens per cage; floor space of 200 cm² per hen). After a 2-week adaptation period, the hens were fed experimental diets for 12 weeks. Throughout the trial, the hens were provided with the same basal diet formulated according to the National Research Council (NRC, 1994) recommendations. While the control group was fed the basal diet, the diets of the other groups were supplemented with boron in different forms (boric acid-sodium pentaborate pentahydrate) and levels (50 mg/kg and 100 mg/kg).

Table. 1 Ingredients, composition of hens diets

Ingredients	**%**
Sunflower oil	3.70
Maize	55.00
Soybean Meal (44%, CP)	22.00
Sunflower Meal (32%, CP)	8.00
Dicalcium Phosphate[a]	2.00
Limestone	8.50
DL-Methionine	0.20
NaCl	0.35
Vitamin and Mineral Premix[b]	0.25
Items	**Analyzed values, %**
Dry Matter	90.6
Crude Protein	17.0
Ether extract	5.20
Crude Ash	13.63
Crude Fiber	4.67
Items	**Calculated values, %**
Metabolizable energy (ME), kcal/kg	2806
Calcium	3.81
Available Phosphorus	0.44
Na	0.17
Cl	0.25
Methionine + Cysteine	0.78
Lysine	0.82
Threonine	0.72
Tryptophan	0.27
Linoleic acid	3.02
Electrolyte Balance (mEq/kg DM)	197

[a]Composition: 240 g Ca and 17.5 g P/kg; [a]Vit+Min. Mineral mixture provides the following nutrients per kg of diet: vitamin A: 12.000.000 IU, vitamin D3: 2.500.000 IU; vitamin E: 30 ppm; vitamin K3: 4.000 ppm, vitamin B1: 3.000 ppm, vitamin B2: 7.000 mg, vitamin B12: 5.000 ppm, vitamin C: 50.000 ppm, Biotin: 45 ppm, folic acid: 1.000 ppm, Fe: 60 ppm, Zn: 60 ppm, Cu: 74 ppm, Se, 150 ppm, Co: 200 ppm, I: 1.000 ppm, Mn: 80.000 ppm.

Lighting was maintained on a 16-hour light and 8-hour dark (16L:8D) schedule throughout the experiment. Feeders were manually replenished daily, and eggs were collected each morning.

At the beginning of the experiment, hens were weighed and placed in cages according to similar live weight and egg yields and treatment groups were formed. Feed intake, egg production and egg weight were measured daily during the trial. Feed conversion ratio and other performance data were calculated weekly. For this purpose, a total of 45 eggs were collected from each group over two consecutive days (in the morning), stored overnight at 4°C, and then subjected to external and internal quality analyses. To determine specific gravity, eggs were weighed after being stored in air for one day, then submerged in water at 22°C and weighed again (Hempe et al., 1988). Yolk color was measured as L*, a*, and b* values using a digital colorimeter (Minolta CR-300). Albumen height was measured with a digital micrometer and calculated using the formula: [albumen height (mm) / ((albumen length (mm) + albumen width (mm)) / 2)] × 100. Yolk index was determined by measuring yolk height and diameter with a digital caliper and applying the formula: [(yolk height / yolk diameter) × 100]. Egg weight (g) and albumen height (mm) were used to calculate Haugh units with the formula: 100 × log (H + 7.57 - 1.7G^0.37).

At the end of the trial, one hen from each subgroup (replicate) closest to the group average was slaughtered, and a total of six right femur and tibia bones were collected from each group. The femur and tibia bones were cleaned of muscle and fat tissues, wrapped in cling film to prevent air exposure, labelled, and stored in double plastic bags at −20°C for later analysis. Before testing, the bones were dried at 60°C for 24 hours and defatted using an ether solvent. For breaking strength measurements, the frozen bones were thawed at room temperature (28°C) for 16 hours. A Stepless Compression Test Machine was used for 3-point bending tests, with each bone centered under a 10 mm diameter probe moving at 0.5 mm/min. To identify the fracture point, 20 mm sections from the dorsal and distal ends of the femur were excluded, 40 mm was subtracted from the minimum bone length, and the remaining length was divided equally using a calliper.

After strength testing, the bones were dried at 105°C for 24 hours, cooled in a desiccator, and weighed. They were then fat-extracted with ether for 24 hours, reweighed, and ashed at 550°C for 24 hours to determine ash weight. The results were calculated as the percentage of ash relative to the dry, fat-free weight, following the method of Elaroussi et al. (1994).

At the end of the trial, bone samples were collected from the femur of one hen per pen per week (n = 45). The samples were dried, ground, and prepared for mineral analysis.

Data were subjected to one-way analysis of variance (ANOVA) using SPSS software (version 18.0; IBM Corp., Armonk, NY, USA). When significant differences were detected, treatment means were compared using Tukey's multiple comparison test at a significance level of $P < 0.05$.

RESULTS AND DISCUSSIONS

Table 2 shows the effects of the experimental diets on egg production, egg weight, feed intake and feed conversion rate. Boric acid and borax pentahydrate supplements increased egg production while reducing feed consumption and improving feed conversion ratio. The highest egg production was observed in the borax pentahydrate (100 mg/kg) group (88.6%, P = 0.015). The lowest feed consumption was also recorded in the borax pentahydrate (100 mg/kg) group (124.8 g/bird/day, P<0.001), and this was associated with the positive effects of boron supplementation on gut health. The best feed conversion ratio was found in the borax pentahydrate (100 mg/kg) group (2.43, P<0.001), which aligns with findings in the literature indicating that boron supports mineral metabolism and enhances feed utilization (Bozkurt et al., 2015; Rossi et al., 2018; Mohammadpour et al., 2016). Recent studies also support the positive effects of boron supplementation on digestive system health and feed efficiency (Akhavan-Salamat & Ghasemi, 2020; Khattak et al., 2018; Oguz et al., 2017).

The highest egg production in the borax pentahydrate (100 mg/kg) group can be explained by boron's ability to improve gut health and enhance feed utilization (Akhavan-Salamat & Ghasemi, 2020). The decrease in feed consumption with increasing boron levels suggests that boron improves intestinal morphology and increases nutrient absorption (Mohammadpour & Kamyab, 2021). The lower feed conversion ratio is attributed to boron's

regulation of calcium and magnesium metabolism, which enhances energy utilization efficiency (Khattak et al., 2018).

The effects of boric acid and borax pentahydrate supplementation on external egg quality traits in hens throughout the study are shown in Table 3.

Boron supplements improved eggshell quality, with the highest shell thickness measured in the borax pentahydrate (100 mg/kg) group (0.379 mm, P = 0.026). This result is consistent with studies showing that boron supports calcium metabolism, enhancing shell density and strength (Kabu & Akosman, 2013; Eren et al., 2012). However, no significant differences were observed among the groups in terms of egg shape index, specific gravity, or shell ratio (P > 0.05), which aligns with some studies suggesting that boron supplementation has limited effects on these parameters (Eren et al., 2004). Recent studies also support the positive effects of boron supplementation on eggshell density and quality (Mohammadpour & Kamyab, 2021).

The effects of boric acid and borax pentahydrate supplementation on internal egg quality traits in hens throughout the study are shown in Table 4.

Table 2. Effects of dietary supplementation with boric acid and borax pentahydrate on productive performance in hens throughout the study (25 to 37 wk of age)

		Boric Acid (mg/kg feed)		Borax Pentahydrate (mg/kg feed)			
Parameters	Control	50	100	50	100	SEM[1]	*P-value*
Egg Production, %	83.4^{b}	85.8^{ab}	86.5^{ab}	86.5^{ab}	88.6^{a}	0.498	0.015
Egg yield, g/hen/day	56.6^{b}	57.8^{ab}	58.8^{a}	57.4^{ab}	59.2^{a}	0.246	0.036
Feed Intake (FI), g/hen/day	138.9^{a}	136.0^{a}	130.8^{ab}	135.5^{a}	124.8^{b}	1.245	<0.001
Egg Weight (EW), g/day	59.1	61.3	60.2	59.8	60.8	0.303	0.156
Feed Conversion Rate (FI/EW)	2.62^{a}	2.56^{ab}	2.48^{bc}	2.54^{abc}	2.43^{c}	0.016	<0.001

^{a,b}Means within each period with different superscript letters are significantly different (P < 0.05).
[1]SEM = pooled standard error of the mean.

Table 3. Effects of Dietary Supplementation with Boric Acid and Borax Pentahydrate on External Egg Quality Traits in Hens throughout the study (25 to 37 wk of age)

		Boric Acid (mg/kg feed)		Borax Pentahydrate (mg/kg feed)			
Parameters	Control	50	100	50	100	SEM[1]	*P-value*
Egg Shape Index	77.6	76.6	78.1	76.6	77.4	0.287	0.488
Specific Gravity	1.18	1.17	1.18	1.18	1.19	0.0005	0.345
Egg Shell Rate	11.3	11.4	11.5	11.7	11.7	0.078	0.614
Shell Thickness, mm	0.342^{b}	0.344^{b}	0.363^{ab}	0.360^{ab}	0.379^{a}	0.004	0.026

^{a,b}Means within each period with different superscript letters are significantly different (P < 0.05).
[1]SEM = pooled standard error of the mean.

Table 4. Effects of Dietary Supplementation with Boric Acid and Borax Pentahydrate on Internal Egg Quality Traits in Hens throughout the study (25 to 37 wk of age)

		Boric Acid (mg/kg feed)		Borax Pentahydrate (mg/kg feed)			
Parameters	Control	50	100	50	100	SEM[1]	*P-value*
Haugh Unit	78.2	79.1	80.5	78.7	80.1	0.459	0.456
Albumen Index	8.99	9.16	9.25	8.93	9.12	0.137	0.956
Yolk Index	44.1	45.7	45.9	45.2	45.2	0.281	0.351
L*	55.1	55.5	55.2	54.5	53.8	0.230	0.118
a**	17.8^{a}	16.9^{ab}	17.1^{ab}	16.2^{ab}	15.0^{b}	0.295	0.020
b***	33.8	32.3	34.0	32.5	33.6	0.345	0.183

^{a,b}Means within each period with different superscript letters are significantly different (P < 0.05).
L*: lightness, a**: redness, b***: yellowness.
[1]SEM = pooled standard error of the mean.

The increase in shell thickness at higher boron levels can be explained by boron's support for calcium and phosphorus metabolism, improving shell mineralization (Kabu & Akosman, 2013). The lack of difference in egg shape index and specific gravity may be due to boron's more pronounced effect on external shell structure compared to internal structure.

Boron supplementation had a limited impact on internal quality parameters, with no significant differences observed between groups in terms of Haugh unit and albumen index ($P > 0.05$). However, as boron levels increased, the reddish hue of the egg yolk decreased ($P = 0.020$), which may be linked to boron's effects on antioxidant enzyme activity (Oğuz et al., 2017; Pradhan et al., 2021). The limited effect on internal quality parameters suggests that boron's influence on gut health and mineral metabolism may not lead to significant changes in egg composition (Bozkurt et al., 2015; Akhavan-Salamat & Ghasemi, 2020). Recent studies also highlight boron's effects on antioxidant mechanisms, supporting the observed changes in yolk color (Khattak et al., 2018; Mohammadpour & Kamyab, 2021). The lack of significant differences in Haugh unit and albumen index suggests that boron primarily plays a role in mineral metabolism rather than directly affecting protein structure (Bozkurt et al., 2015). The limited variations in internal quality parameters indicate that boron's metabolic effects are more focused on eggshell formation rather than egg composition (Mohammadpour & Kamyab, 2021).

The bone mineralization results in Table 5, although not statistically significant, complement earlier findings in the study regarding improved mineral metabolism with boron supplementation - especially in groups receiving 100 mg/kg of either boric acid or borax pentahydrate. Although improvements in tibia and femur strength and mineral content were not statistically significant, the consistent upward trend - especially in calcium content - aligns with previous research (Khattak et al., 2018; Bozkurt et al., 2015) which noted enhanced bone mineralization from boron supplementation. The lack of significance might be attributed to high inter-individual variability or the duration of the study. Overall, the results suggest a potentially beneficial but subtle role of boron in bone mineralization, with more pronounced effects observed in egg production and shell quality. Longer-term studies or older hen populations might show clearer outcomes in skeletal metrics.

Table 5. Effects of Dietary Supplementation with Boric Acid and Borax Pentahydrate on Bone Mineralization in Hens (at 37 wk of age)

		Boric Acid (mg/kg feed)		Borax Pentahydrate (mg/kg feed)			
Parameters	Control	50	100	50	100	SEM[1]	*P-value*
Tibia strength (N)	87.1	89.1	92.6	89.4	88.8	0.541	0.089
Tibia ash (%)	48.2	49.1	48.5	49.0	48.8	0.256	0.632
Ca in Tibia (%)	16.9	17.0	17.2	16.9	17.5	0.296	0.442
P in Tibia (%)	7.6	7.4	7.7	7.5	7.8	0.212	0.096
Femur strength (N)	86.1	85.7	85.7	87.1	86.4	0.381	0.401
Femur ash (%)	45.2	46.5	46.7	45.9	46.8	0.130	0.536
Ca in Femur (%)	22.4	23.6	27.5	26.7	27.0	0.301	0.091
P in Femur (%)	9.2	8.9	9.0	9.3	9.2	0.112	0.381

[a,b]Means within each period with different superscript letters are significantly different ($P < 0.05$).
[1]SEM = pooled standard error of the mean.

CONCLUSIONS

This study explored the effects of boric acid and borax pentahydrate supplementation at different levels (50 and 100 mg/kg) on laying hens. The results showed that 100 mg/kg borax pentahydrate significantly improved egg production, feed efficiency, and eggshell thickness without negatively affecting internal egg quality. While internal traits remained stable, a slight decrease in yolk redness was observed. Bone mineralization results showed positive trends but were not statistically significant. Overall, boron especially as borax pentahydrate proved beneficial for enhancing egg quality and productivity.

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RESEARCH REGARDING THE EFFECTS OF REPLACING SUNFLOWER MEAL WITH FLAXSEED CAKE IN DAIRY COW DIETS ON THE PRODUCTION AND CHEMICAL COMPOSITION OF MILK

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Abstract

This study investigates the effects of substituting 30%, 60% and 100% sunflower meal concentrate mixture with flaxseed meal on milk production and chemical composition, with special emphasis on lipid profile and fatty acid content. The research was conducted over a 30-day period on a herd of 28 Romanian Black Spotted dairy cows, divided into four groups (one control group and three experimental groups). Milk samples were analyzed in an accredited laboratory to determine dry matter, protein, lipid and fatty acid content. The results showed that flaxseed meal supplementation did not negatively affect total lipid or saturated fatty acid content, but significantly increased omega-3 fatty acids and reduced trans and omega-6 fatty acids, thus improving the nutritional quality of the milk. These findings support existing research and highlight the potential of flaxseed cake as a functional ingredient that enhances milk quality for health-conscious consumers. The study opens important perspectives for integrating this nutritional strategy into modern dairy cow farming practices.

Key words*: dairy cows, flaxseed cake, fatty acids, milk, nutrition, sunflower meal.*

INTRODUCTION

Currently, at the national level, milk and dairy products hold an essential place in consumer preferences, being regarded as staple foods in the daily diet. This widespread appreciation is due to the remarkable nutritional composition of milk, which provides the body with all essential amino acids, five different types of proteins, fatty acids, four forms of lactose, as well as a wide range of essential minerals and enzymes (Enea et al., 2023).

Milk is not just a common food item, but a fundamental component of the human diet, with a significant contribution to maintaining health. Dairy products, in particular, are vital sources of nutrients that support the harmonious development of the body and can have a positive impact on overall well-being. Due to its rich content of proteins, lipids, carbohydrates, vitamins, and minerals, milk is recognized as a complex food, indispensable in a balanced and healthy diet (Defta et al., 2023).

An important factor that influences both the quality and nutritional value of milk is the diet of the dairy cows. The composition of milk is directly shaped by the feed the animals consume, with significant effects on its fatty acid profile, vitamin content, and bioactive compounds. Cows nourished with a balanced, nutrient-dense diet, especially one that includes high-quality forages tend to produce milk of superior nutritional quality and improved sensory attributes. In this context, the use of oilseed cakes has gained considerable attention in recent years (Puppel et al., 2012; Puppel et al., 2013).

Oilseed cakes, which are residual by-products obtained after the extraction of oil from oleaginous seeds, represent a valuable resource in animal nutrition. These feed materials are typically rich in proteins, essential fatty acids, fiber, and various bioactive compounds, depending on the type of seed from which they originate (Abedini et al., 2022). When incorporated into the diet of dairy cows, oilseed cakes not only act as nutritionally dense and cost-effective alternatives to conventional feed but also have the potential to improve the nutritional profile of milk, particularly by

enhancing its lipid content with beneficial unsaturated fatty acids.

Moreover, the use of oilseed cakes in cattle feeding practices contributes to sustainable agricultural systems, by reintegrating agro-industrial by-products into the food chain and reducing feed-related waste. Their inclusion supports both environmental and economic sustainability, aligning with contemporary demands for eco-conscious livestock production and functional food development (Oliveira et al., 2016).

Flax has been known and valued for centuries as a source for extracting vegetable oil. One of the by-products of this process is flaxseed cake, traditionally used as animal feed, particularly for cattle. Pressed flaxseed cake retains a significant amount of valuable compounds such as residual lipids, high-quality proteins, soluble fibers, and lignans, which endow it with high potential for extended nutritional and functional applications (Coşkuner & Karababa, 2007; Gutiérrez et al., 2010).

The aim of this research is to investigate the possibility of replacing conventional protein sources in dairy cow feed, specifically sunflower meal, with residues derived from the extraction of extra virgin oils, namely flaxseed cake and to evaluate their impact on milk yield and quality.

MATERIALS AND METHODS

The study was conducted over a 30-day period on a dairy farm located in the south-central region of Romania, involving 28 Romanian Black Spotted dairy cows (Bălțată cu Negru Românească). The animals were divided into four groups, respectively one control group and three experimental groups. Each experimental group underwent a 7-day adaptation period to adjust to the new feed formulation, which included flaxseed cake.

All cows received the same basal diet, consisting of alfalfa hay, corn silage, and a concentrate mixture. The distinction among the groups was made through the composition of the concentrate. In the control group, the concentrate was composed of corn grain, barley, wheat bran, sunflower meal, monocalcium phosphate. In the experimental groups, sunflower meal was replaced with flaxseed cake at inclusion rates of 30%, 60%, and 100%, respectively. All animals had free access to mineral blocks throughout the trial. The concentrate mixture was administered individually during milking.

The samples were taken from the feed components of the rations and concentrate mixtures and sent to the laboratory, where their chemical composition was determined (dry matter – DM, crude protein, calcium – Ca, phosphorus – P). The energy value of the feed (expressed in UFL – feed units for milk) and the protein value (expressed in PDIN – digestible protein in the intestine based on nitrogen, and PDIE – digestible protein in the intestine based on energy) were obtained by calculation.

The milk production of the cows was measured daily. Milking was performed automatically, and milk samples were collected in sterile containers, stored at 4°C, and transported under controlled conditions to the laboratory analyses. To assess the influence of flaxseed cakes on the quality of milk production, milk samples were collected at the end of the experimental period and sent to an accredited food product analysis laboratory in Romania. The following chemical parameters were determined: dry matter (DM); protein; lipids; fatty acids.

For the statistical analysis of the experimental data, Microsoft Excel was used to calculate the arithmetic mean and the standard error of the mean (SEM). The comparison of means between the control group and the experimental groups (30%, 60%, and 100% flaxseed cake) was performed using analysis of variance (ANOVA), followed by the Tukey post-hoc test to verify whether the differences between means were statistically significant.

The software provided the p-values, which were interpreted as follows: $p > 0.05$ (NS – the difference between means is not significant), $p < 0.05$ (* – the difference is significant), $p < 0.01$ (** – the difference is distinctly significant), $p < 0.001$ (*** – the difference is highly significant). In this way, the real impact of flaxseed cake supplementation on the chemical and lipid parameters of cow's milk was determined. The Table 4 present the mean ± SEM values for each parameter, together with the p-values comparing the control group to each experimental group (30%, 60%, and 100% flaxseed cake).

RESULTS AND DISCUSSIONS

To provide a clearer understanding of the feeding structure applied during the experiment groups, regardless of the protein source included in the concentrate mixtures. Table 1 presents the composition and nutritional value of the rations used for both the control group and the three experimental groups. The rations were formulated to ensure comparable levels of energy and digestible protein across all groups, regardless of the protein source included in the concentrate mixtures.

The detailed composition and nutritional values of the basal diet and the concentrate mixtures are shown in Table 1.

Tabel 1. Composition and nutritional value of the rations

Ingredients	kg	DM (Kg)	UFL	PDIN (g)	PDIE (g)	Ca (g)	P (g)
Basal ration							
Alfalfa hay	7.8	6.8	4.9	702	608.4	78	14.8
Ensiled corn	22	5.7	4.6	286	374	26.4	11
Concentrate mixture							
Control group	7.2	88.5	104.8	10302.2	10706.8	306.4	693.3
Experimental group 1	7.2	88.5	106.4	10285.1	10974.7	304.7	684.7
Experimental group 2	7.2	88.5	106.5	10448	11217.6	301	707.2
Experimental group 3	7.2	88.5	106.7	10676.6	11549.2	295.8	738.9
Total ration							
Control group	37	31.4	26.8	2717.6	2735.2	230.8	101.5
Experimental group 1	37	31.4	26.8	2717.6	2735.2	230.8	101.5
Experimental group 2	37	31.4	26.8	2724.8	2771.2	230.5	102.5
Experimental group 3	37	31.4	26.9	2739.2	27922.8	230	104.8

As shown in Table 1, replacing sunflower meal with flaxseed cake at varying inclusion levels did not significantly affect the energy content or digestible protein supply of the rations, thus providing a nutritionally balanced framework for evaluating the experimental effects. While the Table 1 described the overall nutritional values of the rations, Table 2 provides a detailed breakdown of the concentrate mixtures used for each group, emphasizing how the inclusion of flaxseed cake replaced sunflower meal in the experimental diets.

Table 2. Composition of the concentrate feed mixtures in the study groups' rations

Group	Quantity (kg)					
	Corn	Barley	Wheat bran	Sunflower meal	Flaxseed cake	Monocalcium phosphate
Control group	56.5	14.3	9	19.2	0	1
Experimental group 1	56.5	14.3	9	13.5	5.7	1
Experimental group 2	56.5	9.3	14	7.8	11.4	1
Experimental group 3	56.5	2.3	21	0	19.2	1

The gradual replacement of sunflower meal with flaxseed cake in the experimental groups' rations allowed for the investigation of the effects of this change without significantly altering the nutritional balance of the diets. This strategy ensured the necessary consistency between groups and provided an optimal framework for analyzing the specific impact of flaxseed cake inclusion on milk production and composition. To highlight the differences in the nutritional profile of the concentrate feed mixtures, depending on the level of sunflower meal replacement with flaxseed cake, the nutritional values expressed per kilogram of feed are presented in Table 3.

Table 3. Nutritional value of the concentrate feed mixtures used in the study groups' rations

Group	Nutritional value (expressed per kilogram of feed)					
	DM (Kg)	UFL	PDIN (g)	PDIE (g)	Ca (g)	P (g)
Control group	6.36	3.9	465	400	747.4	480.1
Experimental group 1	7.26	4.9	688	563	751.1	488.6
Experimental group 2	7.26	4.9	688	563	751.1	488.6
Experimental group 3	7.26	4.9	688	563	751.1	488.6

The values presented in Table 3 indicate a slight increase in energy and digestible protein content in the concentrate mixtures that included flaxseed cake, without significant changes in calcium, phosphorus, or carotene levels. This nutritional balance between groups is essential for an objective evaluation of the effects of flaxseed cake on milk production and composition.

Throughout the experimental period, milk production remained constant across all groups, including the control group and the experimental groups supplemented with 30%, 60%, and 100% flaxseed cake. The daily yields recorded over the entire experimental period were 22 ± 0.51 l/cow/day (control group), 21.2 ± 0.52 l/cow/day (experimental group 1, 30% flaxseed cake), 21.6 ± 0.48 l/cow/day (experimental group 2, 60% flaxseed cake), and 21.5 ± 0.47 l/cow/day (experimental group 3, 100% flaxseed cake), with no statistically significant differences observed between the groups. These results indicate that the inclusion of flaxseed cake in the cows' diet did not negatively impact milk yield, confirming the stability of production performance while allowing for improvements in milk quality parameters.

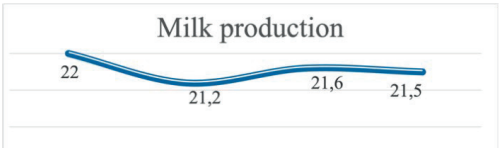


Figure 1. Daily milk production in control and experimental groups

The supplementation of dairy cow rations with linseed cake at levels of 30%, 60%, and 100% generated significant changes in the chemical composition of milk, especially regarding the lipid profile and fatty acid content, as shown in Table 4.

Table 4. Statistical interpretation of the chemical and lipid parameters of cow's milk following flaxseed cake supplementation

Parameter	Control group (mean ± SEM)	Experimental group 1 (mean ± SEM)	p Control vs 30%	Experimental group 2 (mean ± SEM)	p Control vs 60%	Experimental group 3 (mean ± SEM)	p Control vs 100%
Dry matter (%)	12.39 ± 0.31	12.75 ± 0.2 ^{NS}	0.8814	13.08 ± 0.31^{NS}	0.5044	13.52 ± 0.49 ^{NS}	0.1233
Lipids (g/100g)	4.20 ± 0.15	4.33 ± 0.08 ^{NS}	0.8799	4.44 ± 0.12 ^{NS}	0.5315	4.60 ± 0.14 ^{NS}	0.1316
Proteins (g/100g)	3.32 ± 0.10	3.49 ± 0.09 ^{NS}	0.6248	3.65 ± 0.10 ^{NS}	0.1136	3.86 ± 0.10**	0.004
∑ Trans fatty acids (%)	2.28 ± 0.04	2.16 ± 0.06 ^{NS}	0.261	2.05 ± 0.05 **	0.0071	1.90 ± 0.02***	0
∑ Saturated fatty acids (%)	68.04 ± 0.92	68.57 ± 2.43 ^{NS}	0.9987	69.05 ± 1.97 ^{NS}	0.9915	69.70 ± 3.75 ^{NS}	0.9645
∑ Monounsaturated fatty acids (%)	23.15 ± 0.32	23.42 ± 0.23 ^{NS}	0.9268	23.66 ± 0.36 ^{NS}	0.6578	23.98 ± 0.31 ^{NS}	0.2605
∑ Polyunsaturated fatty acids (%)	3.19 ± 0.06	3.23 ± 0.04 ^{NS}	0.9752	3.26 ± 0.10 ^{NS}	0.8847	3.30 ± 0.05 ^{NS}	0.6662
∑ Omega-3 fatty acids (%)	0.48 ± 0.00	0.63 ± 0.01***	0	0.77 ± 0.01***	0	0.96 ± 0.02***	0
∑ Omega-6 fatty acids (%)	2.82 ± 0.04	2.67 ± 0.05 ^{NS}	0.2483	2.53 ± 0.05**	0.0057	2.34 ± 0.07***	0
C10:0 acid (%)	3.67 ± 0.10	3.71 ± 0.11 ^{NS}	0.9894	3.76 ± 0.06 ^{NS}	0.8967	3.81 ± 0.09 ^{NS}	0.7009
C12:0 acid (%)	4.18 ± 0.12	4.24 ± 0.16 ^{NS}	0.9878	4.30 ± 0.16 ^{NS}	0.9146	4.37 ± 0.07 ^{NS}	0.7344
C14:0 acid (%)	12.07 ± 0.24	12.09 ± 0.30 ^{NS}	0.9999	12.11 ± 0.19 ^{NS}	0.9992	12.14 ± 0.12 ^{NS}	0.996
C14:1 acid (%)	1.06 ± 0.02	1.08 ± 0.02 ^{NS}	0.9147	1.10 ± 0.02 ^{NS}	0.5725	1.12 ± 0.03 ^{NS}	0.2353

Parameter	Control group (mean ± SEM)	Experimental group 1 (mean ± SEM)	p Control vs 30%	Experimental group 2 (mean ± SEM)	p Control vs 60%	Experimental group 3 (mean ± SEM)	p Control vs 100%
C15:0 acid (%)	0.87 ± 0.03	0.90 ± 0.04 ^{NS}	0.894	0.92 ± 0.03 ^{NS}	0.6475	0.96 ± 0.02 ^{NS}	0.1766
C16:0 acid (%)	28.75 ± 0.54	28.60 ± 0.54 ^{NS}	0.9974	28.46 ± 0.32 ^{NS}	0.9821	28.27 ± 0.73 ^{NS}	0.9265
C16:1 acid (%)	1.37 ± 0.05	1.37 ± 0.05 ^{NS}	1	1.37 ± 0.03 ^{NS}	1	1.37 ± 0.02 ^{NS}	1
C17:0 acid (%)	0.43 ± 0.01	0.43 ± 0.01 ^{NS}	1	0.44 ± 0.01 ^{NS}	0.8363	0.44 ± 0.01 ^{NS}	0.8363
C18:0 acid (%)	9.56 ± 0.25	9.74 ± 0.31 ^{NS}	0.9644	9.90 ± 0.20 ^{NS}	0.8086	10.11 ± 0.29 ^{NS}	0.4854
C18:1 cis acid (%)	20.71 ± 0.60	20.96 ± 0.63 ^{NS}	0.9935	21.19 ± 0.97 ^{NS}	0.9571	21.49 ± 0.34 ^{NS}	0.8441
C18:1 trans-vaccenic acid (%)	1.72 ± 0.05	1.54 ± 0.03*	0.0495	1.38 ± 0.07***	0.0001	1.16 ± 0.03***	0
C18:2 cis acid (%)	2.46 ± 0.09	2.37 ± 0.05 ^{NS}	0.7226	2.30 ± 0.05 ^{NS}	0.2689	2.19 ± 0.04*	0.0211
C18:3 cis n3 acid (%)	0.48 ± 0.03	0.60 ± 0.01*	0.0191	0.70 ± 0.02***	0	0.84 ± 0.03***	0
C20:0 acid (%)	0.17 ± 0.00	0.15 ± 0.00***	0.0007	0.13 ± 0.00 ^{NS}	0	0.11 ± 0.00***	0
C20:4 n6 acid (%)	0.13 ± 0.01	0.13 ± 0.00 ^{NS}	1	0.14 ± 0.00 ^{NS}	0.3911	0.14 ± 0.01^{NS}	0.3911
C20:5 n3 acid (%)	0.12 ± 0.00	0.11 ± 0.00 ***	0.2716	0.11 ± 0.00 ***	0.2716	0.10 ± 0.00**	0.0055
C4:0 acid	2.02 ± 0.09	2.21 ± 0.03 ^{NS}	0.2804	2.37 ± 0.05*	0.0121	2.60 ± 0.10***	0
C6:0 acid	1.56 ± 0.04	1.73 ± 0.05 ^{NS}	0.0643	1.89 ± 0.03***	0.0002	2.10 ± 0.06***	0
C8:0 acid	1.63 ± 0.03	1.69 ± 0.02 ^{NS}	0.4749	1.75 ± 0.03*	0.0349	1.82 ± 0.03***	0.0006
∑ Omega-9 fatty acids (%)	20.67 ± 0.31	20.78 ± 0.31 ^{NS}	0.9982	20.88 ± 0.54^{NS}	0.9881	21.02 ± 0.60 ^{NS}	0.9491

The mean values and standard deviations of the analyzed parameters are presented in Table 4. Statistical analysis showed that dry matter, lipids, and proteins did not undergo significant modifications at 30% and 60% flaxseed cake compared to the control ($p > 0.05$); however, at 100% flaxseed cake, a significant increase in protein content was observed ($p = 0.004$). This may be attributed to the additional protein contribution from the flaxseed cake, confirming the observations of Huang et al. (2022), who reported similar improvements in milk protein composition following supplementation with flaxseed.

Regarding the fatty acid profile, the data indicate important modifications. The omega-3 fatty acid content increased significantly ($p < 0.05$) in all experimental groups, from 0.48% (control) up to 0.96% (100% flaxseed cake), demonstrating the high efficiency of including this ingredient in cow rations to improve milk quality. Similar results were reported by Caroprese et al. (2010), who showed a significant increase in omega-3 fatty acids in the milk of cows fed flaxseed or flaxseed cake, as well as by Puppel et al. (2023), who reported a 132% increase in linolenic acid (C18:3 n3) and a 194% increase in CLA (C18:2 cis9 trans11) in the milk of organically fed cows supplemented with flaxseed cake.

On the other hand, the trans fatty acid content decreased significantly, from 2.28% to 1.90% ($p < 0.05$). This result is relevant, as trans fatty acids are considered risk factors for human cardiovascular health. These observations are consistent with the research carried out by Oliveira et al. (2021), who showed that supplementation with omega-3-rich vegetable oils reduces the concentration of trans fatty acids in milk. Although Puppel et al. (2023) reported up to a 40% reduction in saturated fatty acids (SFA), especially palmitic acid (C16:0) and myristic acid (C14:0), in this study no significant differences were observed between groups for these parameters ($p > 0.05$).

Although this study did not observe statistically significant increases in monounsaturated fatty acids (MUFA), including oleic acid (C18:1 cis9) ($p > 0.05$), the trend aligns with increases of up to 10% reported by Puppel et al. (2023) after six weeks of flaxseed cake supplementation.

Additionally, the levels of vaccenic acid (C18:1 trans11), an important precursor for CLA synthesis in the mammary gland, increased significantly, confirming the trends reported by Puppel et al. (2023), where a 59% increase was recorded.

Comparisons with recent scientific literature, including studies by Puppel et al. (2023), Huang et al. (2022), Oliveira et al. (2021), and earlier research by Caroprese et al. (2010), validate the consistency of the significant results obtained in this research particularly for protein content, omega-3 fatty acids, and trans fatty acids, and support the hypothesis that flaxseed cake supplementation represents an effective nutritional strategy, applicable both in intensive and organic production systems.

CONCLUSIONS

Overall, the findings of this study clearly demonstrate that flaxseed cake supplementation in the diets of dairy cows, at increasing inclusion levels of 30%, 60%, and 100%, positively influences milk composition, particularly by enhancing the lipid and fatty acid profiles.

The significant increase in omega-3 fatty acids, coupled with the reduction in trans and omega-6 fatty acids, highlights flaxseed cake as a valuable functional ingredient that improves the nutritional quality of milk without negatively impacting general parameters such as total lipids or saturated fatty acids.

This study confirm the potential of flaxseed cake use for producing milk with enhanced functional value, aligning with the growing demands of health-conscious consumers and opening important perspectives for integrating this approach into modern dairy farming practices.

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CHANGES OF THE GLUTATHIONE CONTENT IN THE BLOOD SERUM OF ROOSTERS UNDER THE INFLUENCE OF POLYPHENOLS EXTRACTED FROM NETTLE *(Urtica dioica)*

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Abstract

Oxidative stress has long been implicated in the development and progress of various disorders of living organisms. Glutathione is a natural antioxidant that possesses a major regenerative and detoxifying potential. Glutathione synthesis occurs continuously in almost all cells to maintain redox balance. Ensuring an adequate level of glutathione is vitally important, therefore the role of the glutathione system in maintaining the antioxidant status of the organism is essential. Normally, the formation of free radicals and underoxidized metabolic products occurs continuously during the body`s biochemical reactions. The balance is maintained by antioxidant enzymes that can neutralize molecules with a high oxidative potential. Glutathione is a unique peptide found in the cells of all eukaryotes. This compound plays a leading role in cellular metabolism, actively maintains the redox potential, regulates the detoxification processes of xenobiotics of endo- and exogenous origin, both directly and as a substrate for a number of enzymes. This paper is an analysis of the results obtained from the administration of polyphenols from nettle and their influence on zinc metabolism.

Key words*: glutathione, nutrition, oxidation, polyphenols.*

INTRODUCTION

Intensification of the growth and improvement of farm animals and poultry requires monitoring the state of the body's antioxidant system, otherwise oxidative stress can develop. In order to maintain the functionality of the body's organs and organ systems, it is necessary to stop the development of oxidative stress, through the inclusion of various remedies with antioxidant activity, including those of plant origin. The biochemical structure of herbal remedies is conditioned by their adaptation in enzyme metabolism through evolution, possessing a more pronounced degree of bioavailability compared to antioxidant remedies of synthetic origin (Balan et al., 2024).

The biochemical structure of herbal remedies is close to the structure of metabolites of living organisms, which is conditioned by adaptation by evolution, and correspondingly, these remedies are more easily subject to the influence of fermentative systems, compared to synthetic analogues. In this way, the issue of research and study of new opportunities and phytoprotective sources of natural antioxidants is currently pursued.

Glutathione is the most quantitative intracellular antioxidant, having a role in maintaining redox homeostasis and cellular protection against oxidative stress by neutralizing reactive oxygen species (Forman et al., 2009). It plays an essential role in maintaining the redox state of cells due to its ability to act as a reducing agent.

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) with multiple functions in living organisms (Diaz Vivancos et al., 2010; Sies, 1999). Reduced glutathione (GSH) constitutes an active thiol group in the form of cysteine compounds, which acts as an antioxidant directly by interacting with reactive oxygen species (ROS) and electrophiles, in

particular, acts as a cofactor for various enzymes (Cooper et al., 2011). At the same time, the glutathione content in the intracellular environment has a moderate stability ensured by the cleavage of the peptide bonds formed by the α-carboxyl groups of amino acids by means of intracellular peptidases, but not by the γ-carboxyl groups by carboxylation or decarboxylation reactions, which do not directly involve the peptidases. Furthermore, for the regulation and maintenance of redox balance in cells, reduced glutathione (GSH) and oxidized forms of glutathione (GSSG) act together with other redox compounds, such as nicotinamide adenine dinucleotide phosphate (NADP+) generated by glucose metabolism pathways and protection of cells from oxidative stress (Jones et al., 2011).

GSH is often referred to as the main antioxidant (Bishayee et al., 2010) and being a tripeptide consisting of three amino acids: glutamate, cysteine and glycine plays an essential role in protecting cells against oxidative stress and oxidative damage by maintaining redox homeostasis. GSH is considered as a key element of the antioxidant defense system by neutralizing ROS species and reactive nitrogen species (RNS) thus preventing cell damage and is essential for metabolic processes (Pizzorno, 2014).

Oxidative stress is triggered by the imbalance between the production of ROS and the inability of antioxidant biological systems to eliminate them. In this situation, GSH is the basic redox agent in most aerobic organisms and a fundamental element in fine-tuning oxidative stress. GSH is involved in numerous vital functions, such as free radical scavenging, cysteine delivery, protein thiol status maintenance through redox exchange reactions, modulation of DNA synthesis, processes related to molecular microtubulation, as well as cell immune functions (Lu, 2009). Therefore, GSH plays an important role in cell metabolism, cell differentiation, proliferation, and apoptosis, and reducing ROS and other oxidizing molecules.

The function of ROS and RNS elimination is of great importance for metabolic functioning, and in this vein, GSH can directly bind some ROS species or serve as an essential source of reducing power for certain antioxidant systems (Bray & Taylor, 1993). At the same time, GSH by direct reaction considerably reduces ROS and RNS, but also other radicals, especially HO•, HOCl, RO•, $RO^{2\bullet}$,1 O^2 and $ONOO^-$ and is involved in the detoxification of xenobiotics and products derived from the oxidation of lipidaldehydes (4-hydroxy-2-nonenal as a product of the oxidation of unsaturated fatty acids), facilitating their elimination from the body (Lang et al., 2002). In addition, GSH serves as an electron donor for various antioxidant enzymes, including glutaredoxins, glutathione peroxidases, and glutathione S-transferases, and may be involved in the synthesis of ergothioneine (EGT), another enzyme-catalyzed antioxidant similar to glutathione with a significant role in cellular protection against oxidative stress. In turn, EGT is involved in the chelation of divalent metals (Co, Cu, Hg, Ce, Pt) and in radiation reactions by physically deactivating high-energy molecules through energy transfer (Borodina et al., 2020; Yadan, 2022). At the same time, ergothioneine helps to regenerate glutathione, thus helping to maintain its optimal levels in cells, and the presence of both antioxidants has a synergistic effect, improving the body's ability to cope with oxidative stress.

In the context of the above, the purpose of the present research was to study the possibilities of influencing the glutathione content in the blood serum of roosters under the influence of polyphenolic compounds of natural origin.

MATERIALS AND METHODS

The research was carried out on 10 breeding roosters, which were divided into two batches of five birds each (control and experimental batch). The birds were fed and maintained under identical, analogue investigation conditions, with the same microclimate, technological elements, watering, feeding, veterinary medical assistance, etc. in order to prevent the variation of the sample. The roosters in the experimental batch were administered *per bone* hydroalcoholic extract of polyphenols from nettle (*Urtica dioica*) in a dose of 1 ml with a total antioxidant activity of 33.2 mg gallic acid equivalent per 100 g, and the roosters in the reference batch were administered by the same method with five ml

of saline solution. The well-known methodology of investigation of hematological and biochemical indices in the blood plasma of birds was applied, as well as the determination of the content of free amino acids in seminal plasma and reproductive cells was applied. The sperm from the males was collected by the accepted method of abdominal massage. The semen underwent centrifugation (1000 rpm/min, 10 min) to separate the seminal plasma from the reproductive cells. The reproductive cells subsequently followed the technological process until the amino acid content was determined. The seminal plasma underwent double centrifugation under the conditions mentioned for complete removal of sperm debris from the plasma. The seminal plasma was transferred and stored in the freezer at a temperature of -45°C with subsequent transmission to the profile laboratory for the determination of amino acid content.

The biometric processing of the experimental results was carried out in accordance with the generally accepted methodology. The results are expressed as a mean ± standard error of mean with the determination of significance between the control and experimental lots.

RESULTS AND DISCUSSIONS

Glutathione plays an essential role in maintaining cellular health and oxidative homeostasis and is one of the most important antioxidants in the body. It helps neutralize ROS generated during cellular metabolism, thus preventing oxidative stress that can negatively affect the reproductive health of roosters. Moreover, adequate glutathione levels are essential for the optimal functioning of the testes and sperm production, and also have a significant role in regulating metabolism and supporting the immune system essential for the overall health of breeding roosters and for reproductive success.

Monitoring glutathione levels and ensuring optimal conditions for its production can help improve the reproductive performance of birds. Therefore, initially the value of glutathione concentration indices in the blood serum of breeding roosters was investigated. The results of the research are presented in Table 1.

The data in Table 1 show significant changes in gamma-glutamyltranspeptidase (G-GTP) in the experimental batch compared to the reference batch. The G-GTP content is 11.79±0.59 u/L for the experimental batch compared to 9.33±0.40 u/L in the control batch ($P<0.05$), which demonstrates the influence of the polyphenol extract compounds on these indices.

Table 1. Content of gamma-glutamyltranspeptidase and glutathione transferase in the blood serum of breeding roosters

Groups	G-GTP, u/L	GST, nM/Sl
Experimental	11.79±0.59*	33.00±0.56*
Control	9.33±0.40	19.21±0.38

Note:
*The differences are statistically true between the experimental and control group ($P<0.05$);
G-GTP - gamma-glutamyltranspeptidase;
GST - glutathione transferase.

As a result of the fact that G-GTP catalyzes the transfer of the γ–glutamyl group of peptides and its major content at the level of the cytoplasmic membrane of cells, it is worth mentioning that the increase in its content beneficially influences the oxidative metabolic processes inside the cell. At the same time, the luminous surface of cells with secretory and absorbent functions is especially rich in G-GTP, which influences the extracellular oxidative metabolism of the cell. At the same time, G-GTP is the only enzyme that cleaves significant amounts of GSH and GSH conjugates within the γ-glutamyl cycle (GSH is transported to the extracellular surface of the membrane, where it is separated by G-GTP into cysteinyl-glycine and γ-glutamyl residues, which are transferred to other amino acids) (Deponte, 2013). An important property of glutathione refers to the direct blocking of ROS and intervenes as a redox cofactor for various antioxidant enzymes (glutaredoxins, glutathione peroxidases, glutathione reductase and glutathione S-transferase).

Another studied derivative of glutathione is GSH also because its reactivity with proteins, molecular structures and xenobiotics *in vivo* can be decreased (Deponte, 2017), and the development of GSH-dependent reactions are accelerated by glutaredoxine enzymes that can influence oxidation processes.

In this context, the data in Table 1 show a significant increase in glutathione transferase (GST) content. The value of this index was 33.00±0.56 nM/sL and 19.21±0.38 nM/sL, corresponding in the experimental and reference group ($P<0.05$). Therefore, the increase in the amount of GST in the experimental batch can be correlated with the fact that GST catalyzes conjugation reactions with metabolites, chemicals and metals, or if GST was not depleted during the isomerization and dehalogenation processes, as well as in reactions where GSH can be oxidized by GSH-dependent peroxidases, -thiol transferase, -dehydroascorbate reductase (Deponte, 2013; Deponte, 2017). Glutathione transferases also participate in the binding and transport processes of molecules due to their non-catalytic ligand properties (Schwartz et al., 2018), as well as interacting with proteins, modifying their activity through glutationylation or swallowing (Kalinina & Novichkova, 2021; Sing & Reindi, 2021).

Regarding the interpretation of the results obtained of the researched compound, it is possible to mention that glutathione is maintained in the mitochondria in its reduced form (10-15% of the total cellular GSH) in a concentration of 5-10 mM (Kojer et al., 2012). At the same time, mitochondria do not contain the enzymes necessary for GSH biosynthesis and mitochondrial GSH, respectively, must be imported from the cytoplasm (Scire et al., 2019). Moreover, glutathione has a negative charge at physiological pH and cannot freely cross the lipid bilayer, therefore the outer and inner mitochondrial membranes are provided with vector transporters and channels for GSH absorption. The outer mitochondrial membrane is rich in pores, which form channels through the lipid bilayer and allow diffusion between the intermembrane space and the cytosol of molecules smaller than ~5 kDa, including glutathione (Scire et al., 2019). Kojer demonstrated that glutathione bases in the intermembrane space and cytosol interact through porins (Kojer et al., 2012). This metabolic pathway is widespread in all biological systems and is involved in the catalyzation of the conversion of 2-oxaldehyde to 2-hydroxy acid by interaction with S-2-hydroxyacylglutathione, as well as in the cellular detoxification of α-cetoldehydes produced as a result of glycolysis contributing to the synthesis and metabolism of complex biological and chemical molecules.

The mitochondrial content of GSH is maintained in constant concentration as a result of its transport from the cytosol through two systems, one by increased manifestation produced by adenosine triphosphate (ATP), the other by low influence by ATP and adenosine diphosphate (ADP) with involvement in processes of reduction of oxidative components and in the regulation of the redox state of the cell (Martensson et al., 1990). Regarding other intracellular organelles (endoplasmic reticulum) it has been demonstrated the existence of a transport system that allows selective permeability and transformation of GSH into oxidized form of glutathione (GSSG) (Chakravarthi & Bulleid, 2004) and subsequently at this level GSH participates in the reduction of the activity of isomerases responsible for catalyzing the constitution of disulfide bonds in proteins (Chakravarthi & Bulleid, 2004). Therefore, in the lumen of the endoplasmic reticulum a constant production of GSSG takes place under the influence of the use of GSH that maintains oxidoreductases in their reduced form. Subsequently, through the protein-conducting channels, GSSG by facilitating diffusion is transported to the cytosol (Ponsero et al., 2017), where it undergoes reduction under the action of the enzyme glutathione reductase (Lu, 2013; Chakravarthi & Bulleid, 2004; Ponsero et al., 2017). This process plays an essential role in maintaining cellular redox balance and protecting cells against oxidative stress. Specifically, when cells are subjected to oxidative stress, GSH is oxidized to form GSSG and then GSSG can be reduced back to GSH by the enzyme glutathione reductase, using nicotinamide adenine dinucleotide phosphate (NADPH) as a coenzyme.

The study continued by determining the content of free amino acids in the reproductive material of birds that contribute to the metabolism and presence of glutathione in optimal concentrations in this biological fluid. In particular, for preservation and detoxification with a strategic importance for maintaining life and preserving genetic potential. The research

involved the seminal plasma and reproductive cells of breeding roosters. The results of the research are presented in Table 2.

Table 2. Free amino acid content in seminal plasma and reproductive cells of breeding roosters

Amino acids	Seminal plasma, (mcm/100 ml)	Reproductive cells, (mcm/100 g)
Cysteic acid	22.04±0.34	0.66± 0.04
Glutamic acid	207.54±17.36	7.57±0.22
Glutamine	757.26±42.11	21.75±0.18
Glycine	37.56±2.01	8.88±0.08
Cysteine	4.37±1.04	1.56±0.07
Isoleucine	10.98±1.58	1.55±0.11
Leucine	16.64±2.80	2.25±0.14
Proline	56.90±3.58	6.66±0.02

Table data show that 8 free amino acids have been determined. The results correlate with the existing ones that for the seminal fluid of the rooster it is characteristic that the largest share of the content is represented by glutamine which constitutes 757.26±42.11 mcm/100 ml. Also, the presence of glutamic acid (207.54±17.36 mcm/100 ml) was also determined in major quantities. The content of these two components prevails 6.5 times the total amount of all other amino acids investigated. The content of the latter is increased in the range from 4.37± 1.04 mcm/100 ml for cysteine to 56.90± 3.58 mcm/100 ml for proline.

In the reproductive cells, the same amino acids were determined, but in a significantly smaller amount. The data in the table show that the highest share in spermatozoa is also held by glutamine with a concentration of 21.75± 0.18 mcm/100 g, followed by compounds with practically identical amounts (glycine 8.88±0.08 mcm/100 g, glutamic acid 7.57± 0.22 mcm/100 g and proline 6.66± 0.02 mcm/100 g).

Free amino acids, especially those with reactive functional groups (containing the thiol-SH group) act as antioxidants against ROS and participate in repair and detoxification, and their interaction with glutathione in the oxidation process is essential for cellular protection against oxidative stress. Glutathione reduces reactive oxygen species or other dangerous molecules, becoming GSSG and in this process, the thiol (-SH) group of GSH plays a central role. Furthermore, free amino acids, especially those with thiol groups, participate in reduction reactions or can contribute to the regeneration of GSH from GSSG and thus maintaining redox balance.

Some glutathione restoration mechanisms are maintained by the activity of glutathione-dependent enzymes by releasing cysteinyl-glycine dipeptide that undergoes cleavage into cysteine and glycine by specific Cys-Gly peptidases and 5-oxoproline that is converted to glutamate by ATP-dependent 5-oxoprolinase (Bachhawat & Yadav, 2018). Further the Cys-Gly dipeptide is cleaved by dipeptidase in Cys and Gly with emigration to the cytosol where it participates in the synthesis of proteins and GSH (Bachhawat & Yadav, 2018), and the amide combination between glutamine γ-carboxyl and amino cysteine units prevents the degradation of GSH by circulating serum and cellular peptidases (Lieberman et al., 1996). The process continues with the cellular absorption of Cys, Gly and glutamate units that serve as precursors for the intracellular synthesis of GSH.

GSH in subcellular structures is distributed as follows, in the cytosol (80-85%), mitochondria (10-15%) and endoplasmic reticulum (5-10%) (Giustarini et al., 2015). There is research that has shown that in the endoplasmic reticulum the total GSH content sometimes exceeds the total cellular GSH content (Birk et al., 2013), but these concentrations of GSH, including GSSG depend on the cell type, the subcellular compartment and the individuality of the organism. Consequently, the total glutathione concentration and the ratio of GSH to GSSG are directly proportional to the redox capacity of the variable system of these compounds in tissues and organisms (Kojer et al., 2012). At the same time, cytoplasmic GSH influences its diffusion through nuclear pores (Schafer & Buettner, 2001), where it signals oxidation at the proliferative, epigenetic and cell cycle levels (Markovic et al., 2007).

In the context of the above, the interaction between free amino acids and glutathione plays an essential role in the protection of cells against oxidative stress. Free amino acids,

especially those with reactive functional groups, participate in ROS neutralization reactions, helping to maintain redox balance, and GSH acts as a primary antioxidant, reducing ROS and regenerating other antioxidant molecules. This interaction is essential for detoxifying free radicals and preventing damage to cellular components, such as lipids, proteins, and DNA. In addition, free amino acids can be involved in the synthesis and regeneration of glutathione, thus facilitating the endogenous antioxidant system. Therefore, the close collaboration between free amino acids and glutathione is fundamental for maintaining oxidative balance, preventing oxidative stress and supporting cellular health.

CONCLUSIONS

Glutathione is an essential antioxidant in the body of roosters, having a fundamental role in protecting cells against oxidative stress, which can lead to damage to DNA, proteins and lipids, and by neutralizing ROS, glutathione contributes to maintaining the integrity and optimal function of tissues, including the reproductive system.

The interaction between free amino acids and glutathione helps prevent protein, lipid and DNA damage caused by free radicals, contributing to the maintenance of cellular health, and in particular, free amino acids with thiol groups, actively participate in the processes of glutathione reduction and regeneration, facilitating the neutralization of oxidative stress and maintaining the redox balance in the cell. This interaction is fundamental to the body's antioxidant defense mechanisms.

Adequate levels of glutathione ensure the health of the testicles and sperm, and glutathione's antioxidant activity is significant for increasing fertility, reducing oxidative stress, and promoting efficient and sustainable reproduction in roosters.

Polyphenol extract significantly influences the content of free amino acids in seminal plasma compared to their content in reproductive cells, which denotes about maintaining optimal metabolism with multiple evolutionary functionalities of reproductive cells at all stages of spermatogenesis, including maturation, empowerment and achievement of the ultimate goal of egg fertilization.

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REPRODUCTIVE PERFORMANCE AND INTENSITY OF USE OF PRODUCTIVE POTENTIAL IN COWS

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Abstract

The reproductive performances (SP - service period, CI - calving interval, Insemination index, percentage of fertilization at first insemination) and the intensity of use of milk production potential of Holstein breed (HL) cow population were studied. Change of level of milk productivity cows in the direction of increase or decrease of 1000 kg leads accordingly to decrease or increase the indicators of reproductive function. The reproductive performance indicators of cows in herds with milk productivity from 4000 to ≥ 8000 (SRL "Holstein") and from 6000 to ≥ 10000 kg and more (SRL "Doksancom") revealed two peaks in productivity levels - from 5000 kg milk and from 8000 kg milk. Increasing the service period contributes to increasing the milk yield per normal lactation, but this is not rational because it decreases the average amount of milk per head/day by about 8.1-19.2%, depending on the level of productivity.

Key words*: Holstein breed cows, milk productivity, reproductive performances, use of productive potential.*

INTRODUCTION

The specialized literature has a rich register of data on the study of the relationships between the performance of milk productivity in cows and reproductive indices (Baláš et al., 1994; Moroz et al., 2009; Perfilov et al., 2015; Kuznetsov and Revina, 2017) a single opinion on this subject still does not exist, it still remains in the sights of researchers and specialists of profile.

To confirm or deny this opinion, the purpose of our research was to study the main indicators of reproductive function, their relationship with the level of milk productivity and the intensity of use of the genetic potential of cows.

MATERIALS AND METHODS

In order to achieve the objective, it was experimented with the population of Holstein cows, exploited within the production activity from SRL "Holstein", Roshkani village, Anenii Noi district, SRL "Doksancom", Tomai village, Chiadâr-Lunga district with milk productivity from 4000 to 8000 kg and from 6000 to 10000 kg and more.

To achieve the goal, the following operational objectives were set:

- evaluation of the main reproductive indices: days from calving to the first insemination; days from first insemination to fertilizing insemination; the length of the service period; insemination index; percentage of fertilization at first insemination; the number of inseminations per conception, the number of inseminations per cow;
- reproductive capacity of cows with different milk productivity;
- the relationship between the duration of service period and the intensity of use of the cows productive potential.

For establish the impact of milk productivity on indices of the reproductive function of cows, they were divided into depending on the level of milk yield for normal lactation with a class interval of 1000 kg: <4000; 4001-5000; 5001-6000; 6001-7000; 7001-8000 kg; 8001-9000 kg; 9001-10000 kg; more ≥ 10001 kg. The studies were carried out by comparing groups of animals in terms of reproductive abilities at different levels of productivity.

For insemination, we used frozen sperm of bulls from the Republican Center for Reproduction and Improvement (village Maximovca, Anenii Noi district), conforming to GOST 26030-83 with an activity after thawing not less than 4.0 points, a number of

progressively motile spermatozoa in a dose - 12 million and sperm survival at 38ºC for more than 5 hours.

The material for the research served the data of zootechnical accounting (card form T-2; card form T-10) and the information of data base of the farms.

Statistical processing and biometric analysis of the obtained data were carried out according to the generally accepted methods of variation statistics (Grosu, 2005) using the MS Excel-2010 analysis software package. The reliability of the difference between the mean values of the signs was determined by Student's td-test.

RESULTS AND DISCUSSIONS

In Table 1 are presented the mean days from first insemination to conception, number of inseminations per conception, and total number of inseminations, with relation to the length of the interval between insemination.

Table 1. Mean number of days from first insemination to conception, number of inseminations per conception, and number of inseminations as ranged by ten-day periods of the insemination interval of Holstein breed cows

Specification	Length of interval between insemination, days												
	≤50	51-60	61-70	71-80	81-90	91-100	101-110	111-120	121-130	131-140	141-150	≥151	Total
Cows that conceived													
Days from first insemination to conception	72.1	43.29	41.14	35.77	36.35	30.04	31.07	38.96	37,00	41.54	40.00	12.42	36.08
Cows, heads	11	37	64	68	71	58	52	31	20	20	18	26	476
Inseminations per conception	2.52	2.15	2.47	2.25	2.28	2.09	2.16	2.25	1.28	1.95	2.0	1.51	2.08
Conceived on first insemination, %	30.0	38.55	41.2	48.97	46.75	44.88	47.72	40.05	35.44	46.52	44.4	67.6	54.25
Cows that have not conceived, %	7.13	6.68	5.98	5.85	5.80	5.52	4.23	4.70	4.45	4.15	3.00	3.43	5.78
/ heads	2	4	5	7	8	7	5	3	4	2	1	3	51
Totals cows, heads	13	41	69	75	79	65	57	34	24	24	20	29	527
Inseminations per conception	2.93	2.71	2.40	2.38	2.24	2.23	2.28	2.34	2.54	2.20	1.69	1.78	2.27
Per cent conceived on first insemination, %	29.70	38.95	40.18	49.55	43.65	43.50	47.75	37.95	28.15	42.45	40.00	66.50	52.9

The interval between inseminations ranged from ≤50 to ≥151 days. There were only 13 below 50 days and only 129 above 150 days, of which 14 were above 160 days.

The obtained results attest that the average length of interval from first insemination to conception in the contingent of cows that were first inseminated within 50 days or less after calving is significantly longer compared to those with the length of longer time intervals of first insemination.

At the same time, there are no significant differences between the means regarding the duration of the interval from the first insemination to conception in the sequence of the length of insemination interval.

In Table 2 are shown the analyses of variance for days from first service to conception, for services per conception, and for all services.

Table 2. Analyses of variance for days from first insemination to conception, services per conception, and all inseminations of Holstein breed cows

Specification	Cows that conceived				All cows	
	Days from first insemination to conception		Inseminations per conception		All inseminations per cows	
	Cows	δ^2	Cows	δ^2	Cows	δ^2
Between ten-days interval of inseminations	13	3.099	13	1.11	13	2.76
Within ten-days interval of inseminations	462	2.098	462	2.28	513	2.95

Analysis of variation for days from first service to conception, inseminations per conception, and all inseminations per cow, demonstrates that in no case is the variation between groups with ten-day insemination intervals significant at a probability as low as 0.05.

In spite of the general lack of significance of the between-group mean squares, one should not conclude that the length of the insemination interval is not associated with the length of the interval from first insemination to conception, with inseminations per conception, or with total number of inseminations per cow. The difference associated with the different lengths of insemination interval could be confounded with differences in management. Such differences are likely to be small in the data, because a concerted effort is exerted to standardize management practices at zootechnical holdings and the field non-return rates were approximately equal.

The following table presents the results of the study of reproductive qualities of cows exploited in SRL "Holstein" with a milk productivity level of 4000 to 8000 kg (Table 3). We have established that with an increase in the level of milk yield, from 4000 to 8000 kg, a significant deterioration in fertility after the first insemination was observed in cows starting with a milk yield of more than 5000 kg. Thus, in animals with a milk yield 8000 kg and more, it was 36.4±9.1%, which is 30.1% lower, of 1.6 times lower ($p \leq 0.01$) than in cows with a productivity of ≤ 4000 kg (66.5±6.5%).

Table 3. Reproductive qualities of Holstein breed cows
(SRL "Holstein" with a milk productivity level of 4000-8000 kg)

Milk yield, kg	Cows, heads	Period from calving to first insemination, days	Service period, days	Insemination Index	Conseived from first insemination, %	Period leght from first to fertile insemination, days
≤ 4000	46	55.8 ± 4.2	95.0 ± 10.9	1.6 ± 0.15	66.5 ± 6.5	30.4 ± 7.6
4001-5000	105	64.7 ± 3.3	113.5 ± 10.9	1.6 ± 0.05	56.3 ± 5.4	37. ± 6.7
5001-6000	129	69.5 ± 2.6	129.8 ± 5.8	1.9 ± 0.05	47.2 ± 5.7	52.7 ± 5.8
6001-7000	102	73.2 ± 3.4	14.2 ± 6.8	1.9 ± 0.1	43.2 ± 4.5	57.1 ± 7.1
7001-8000	75	85.6 ± 4.7	152.0 ± 8.4	2 ± 0.1	38.9 ± 5.6	60.4 ± 9.7
≥ 8001	36	80.6 ± 3.8	141.5 ± 9.8	2.1 ± 0.2	36.4 ± 9.1	55.6 ± 6.8

At the same time, the service period increased by an average of 57.0 days, the period from calving to the first insemination by 29.8 days, and the period from the first to fertile insemination by 27.6 days. The results of the analysis showed that the best fertility results after the first insemination are observed in cows with milk productivity more than 7000 kg, inseminated 85.6 days after calving..

A study of the reproductive capacity of cows in a herd with productivity from 6000 to ≥10 000 kg and more (Table 4) showed that the tendency of a decrease in the reproductive function of dairy cattle with an increase in the level of milk productivity persists. The received data are consistent with the information of other researchers (Gabor, 2008).

Table 4. Reproductive qualities of Holstein breed cows SRL "Doksancom"
with a milk productivity level of 6000-10000 kg)

Milk yield, kg	Cows, heads	Period from calving to first insemination, days	Service period, Days	Insemination Index	Period from first to fertile insemination, days	Conseived from first insemination, %
≤6000	58	88.0 ± 5.0	126.3 ± 8.8	1.9 ± 0.16	34.5 ± 7.1	47.8
6001-7000	60	92.6 ± 6.8	132.6 ± 9.9	2.0 ± 0.16	37.6 ± 8.1	43.3
7001-8000	102	82.4 ± 3.0	136.8 ± 5.6	2.2 ± 0.09	52.4 ± 4.9	42.2
8001-9000	148	88.4 ± 4,3	137.4 ± 5.9	2.2 ± 0.10	50.3 ± 4.9	35.8
9001-10000	48	86.5 ± 3,8	153.8 ± 7.9	2.6 ± 0.10	63.8± 7.4	32.4
≥10001	36	88.2 ± 4.8	178.7 ± 11.5	3.0 ± 0.25	71.4 ± 1.4	27.6
Means	452	86.8 ± 1.8	148.4 ± 5.8	2.3 ± 0.06	52.6 ± 2.7	38.1

The obtained data show that, the most pronounced decrease in the conceived insemination of cow is observed with an increase in the level of milk productivity of more than 8000 kg. Other indicators of reproductive function also deteriorate: the insemination index increases (sperm consumption per fertilizing insemination increases by 30% or more), the service period is extended by 11.1-52.4 days.

Thus, with an increase in the level of milk productivity, a decrease in the fertilization rate

of cows, an extension of the service period, and an increase in the insemination index are observed. A change in the level of milk productivity of cows towards an increase or decrease by 1000 kg leads, respectively, to a decrease or increase of indicators of the reproductive function.

The service period is one of the indicators that characterize the efficiency of the reproductive state and an essential component of other indicators as the duration of lactation and also the calving interval. The reproductive function of cows often fails with high productivity and low energy consumption with feed after calving (Dushkin, 2008).

For the deeper disclosure of the particularities of the relationship between the lenght of the service period and intensity of of use of productive potential in cows, we experimented with a herd of 287 Holstein cows, operated under identical technological conditions within SRL "Doksancom" and divided into six classes according to the level milk productivity (Table 5). The analysis of the results obtained regarding the relationship between the legth of service period and the average daily milk production per lactation demonstrates that, in all subclasses, simultaneously with the increase of the first variable, the second one is decreasing. In cows with a milk yield in the range of 7000-8000 kg, simultaneously with the increase in the legth of service period from less than 100 days to 121-160 days, the average daily milk yield decreased by 8.2%. In cows with milk productivity in the range of 8001-9000 kg, in the same service period range, the average daily milk yield decreased by 7.3%. With the further increase of the service period to more than 200 days, the average daily milking decreases by 17.4% compared to the service period lenght of up to 100 days. In the next class (9001-10000 kg), at service period duration of at least 200 days the average daily milking decreased by 12.5% compared to the situation where service period is up to 100 days.

Table 5. The intensity of use of cows according to the duration of the service period (Holstein breed cows, SRL “Doksancom”)

Milk productivity classes (kg)	U.M.	Lenght of service period, days					
		≤ 100	101-120	121-160	161-180	181-200	≥ 201
7000-8000	kg/day	24.3 ± 0.64	23.4 ± 0.39	22.3 ± 0.96	-	-	-
	%	100	96.3	91.8	-	-	-
8001-9000	kg/day	27.7 ± 0.86	26.5 ± 1.02	25.6 ± 1.13	24.5 ± 0.92	23.6 ± 1.05	22.9 ± 1.27
	%	100	95.7	92.7	88.4,0	85,2	82.6
9001-10000	kg/day	31.2 ± 0.85	30.4 ± 0.67	29,6 ± 1,09	29.4 ± 0.56	28.3 ± 0.79	27.3 ± 1.17
	%	100	97.4	94.9	94.2	90,7	87.5
10001-11000	kg/day	34.4 ± 0.41	32.8 ± 0.55	31.6 ± 0.44	30.0 ± 0.74	29.1 ± 0.85	27.8 ± 1.35
	%	100	95.3	91.7	92.6	87.2	80.8
11001-12000	kg/day	-	37.3 ± 1.08	36.2 ± 0.88	33.8 ± 0.42	33.0 ± 0.97	31.4 ± 1.14
	%	-	100	97.1	90.6	88.5	84.2
≥ 12001	kg/day	-	-	41.2 ± 0.84	39.6 ± 0.61	39.4 ± 0.68	34.9 ± 2.64
	%	-	-	100	96.1	95.6	84.7

In cows with the level of milk productivity 10001-11000 kg milk, the daily milk yield at the maximum duration of service pereiod decreased by 19.2%. In the following productivity classes (11001-12000 kg), a decrease in the average daily milking by 15.8% and by 15.3% in the last productivity class (≥ 12001) is attested, compared to the indices established in these variants when the service period lenght was in the range of 101-120 and 121-160 days.

This analysis convincingly demonstrates that under similar housing, feeding and operating conditions of cows lenght of sarvices period affects milk production differently in cows. If the average daily milk production of cows per lactation under the conditions of a service eriod of up to 100 days is considered 100%, when increasing it to at least 201 days the average daily milk production decreased by up to 19.2%. However, milk losses caused by the increase in the service period due to late fertile insemination in cows will cause significant damage to the farm, because the maintenance and nutrition costs of cows with a low reproductive level are the same as for cows with optimal reproductive indices.

Similar results were reported by researchers (Fariniuk and Abylkasymov, 2016).

Increasing the duration of the service period and the interval between calvings negatively

influences the reproductive capacity of cows (Figure 1).

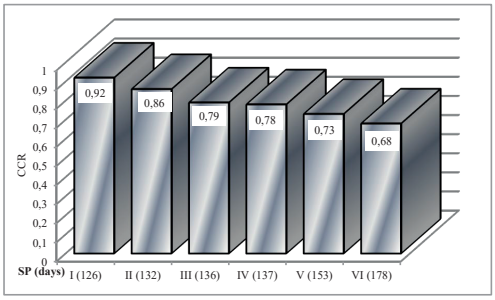


Figure 1. The relationship between the length of service-period and the coefficient of utilization of the reproductive capacity of the female herd (Holstein breed cows, SRL "Doksancom")

Analyzing the obtained data, it is obvious that the coefficient of utilization of the reproductive capacity of cows is in decline as the length of the service period increases, the established differences are statistically inauthentic. The best result was obtained in animals from the first variant, in which the lenght of the service period is statistic authentically higher compared to the variant with the longest service period.

In addition, the increase in the lenght of the service period and the interval between calving negatively influences the reproductive capacity of the cows which is on the dicrease as the milk productivity increases, a fact that even in the current year will decrease the birth rate per herd (Granaci et al., 2023).

Both a small number of inseminations per conception and a short interval from first service to conception are important items and are to be desired by a dairy farms, but are not the only items to be considered in recommending optimunl iuterval from calving to first insemination.

It should be remembered that at present under practical conditions artificial reeding organizations would be interested in a low number of inseminations per conception, as this would be less expensive to the organizations where second and third services are given free of charge, when the first or the first and second inseminations fail to get the cow non-conceived.

Thus, it is likely that artificial breeding organizations would recommend a relatively long service interval. On the other hand, a shorter service interval might result in more first inseminations per cow, since the calving intervals would be shorter, thus producing more income for artificial breeding organizations. Artificial breeding associations would probably be more interested in total number of services as related to insemination interval rather than services per coneption because, at the time of first insemination, no one really knows which cows will conceive and which will not. As shown Moroz et al. (2009), 0.56 less inseminations can be expected for each 100 days of increase in the length of the service period.

The interval from calving to conception defined as service period, should definitely be considered, as it and the length of the gestation period determine the length of the calving interval. Most of the variation in calving interval is brought about by variation in the sevice period, because the variation in the length of the gestation period is relatively small.

The researches (Ulimbashev & Khuranov, 2017; Gorelik et al., 2023) have presented data which indicate that high-producing cows require a considerably increased number of services per conception. These data could be biased, in that there would be a tendency to be more patient with high-producing cows and allow them more chance to conceive than would be the case with low-producing cows. It is also possible that differences in sperm fertilizing ability are confounded with production levels. In addition, it is probable that thos cows requiring more inseminations have a longer period from calving to conception and, thus, less effect of pregnancy on lactations.

Starting from the known fact that the permissible limits for the duration of the service period, it is recommended to be according to the level of milk production in cows

CONCLUSIONS

The relationship between the insemination interval and the interval from the first insemination to conception, as well as between the number of inseminations required for conception and the interval from the first insemination to conception has not been established.

Change of level of milk productivity cows in the direction of increase or decrease by 1000 kg leads accordingly to decrease or increase the indicators of reproductive function.

The reproductive performance indicators of cows in herds with milk productivity from 4000 to ≥ 8000 and from 6000 to ≥10000 kg and more revealed two peaks in productivity levels - from 5000 kg milk and from 8000 kg milk.

Increasing the service period contributes to increasing the milk yield per normal lactation, but this is not rational because it decreases the average amount of milk per head/day by about 8.1-19.2%, depending on the level of productivity, what negatively influences the intensity of realization of the genetic potential of cows.

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PREANESTHETIC GUIDELINES IN SHEEP: ENSURING WELFARE AND SAFETY IN EXPERIMENTAL RESEARCH

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Abstract

The aim of this study was to identify the important preanesthetic factors that could improve anesthesia management in research sheep, focusing on reducing critical complications like regurgitation and aspiration of ruminal contents during general anesthesia, which can lead to serious respiratory risks and potential fatality. Our study was conducted on a group of 13 sheep (Ovis aries), 2-4 years old, selected for different surgical procedures, during September 2022-June 2024 at the Faculty of Veterinary Medicine of Bucharest. For the entire group the protocol involved a 24-hour fasting period, allowing water access until the premedication stage to ensure hydration. A comprehensive clinical evaluation was performed to identify any underlying health issues, and sedatives, along with prokinetic agents, were administered to reduce stress and encourage gastric emptying. Continuous monitoring of vital signs and behavior ensures the stability of the animals during surgical procedures. By following the comprehensive 7-step preanesthetic guidelines, the risk of regurgitation and aspiration, along with other risks can be significantly reduced, thereby improving sheep welfare and safety during general anesthesia.

Key words: *guidelines, preanesthesia, research, sheep.*

INTRODUCTION

Several factors come into play when considering the suitable animal model for experimental research, including anatomy, physiology, and genetic similarity to humans. Each animal model has specific strengths and limitations depending on the area of research.

Pigs have remarkably anatomical and physiological similarity to humans, especially in terms of the gastrointestinal tract, cardiovascular system, skin, and kidneys and are widely used in heart disease studies and surgical research, including organ transplantation, wound healing studies or metabolic studies (Moldovan et al., 2022). Pigs are not easy animals to handle, especially large ones (Costea et al., 2023). While rats are valuable in understanding basic biological mechanisms, their small size raises many anesthetic and surgical risks (Costea et al., 2015) and translating findings to human medicine sometimes requires validation in larger models like pigs or sheep.

Sheep (*Ovis aries*) are a valuable animal model for experimental studies, especially those involving surgical procedures. Their body weight, bone size, and bone-healing potential are similar to humans (Reinholz et al., 2004), making them useful for assessing biomaterials in bone regeneration and the integration of dental implant systems in dentistry, orthopedics, cardiovascular and respiratory system studies (Sartoretto et al., 2016). Since these procedures can cause anxiety and pain in the animals, they should be performed under general anesthesia.

Regurgitation and aspiration of ruminal contents represent significant complications during general anesthesia in sheep. These events can result in severe respiratory problems and even death. It is essential to implement appropriate preanesthetic protocols to minimize these risks. Due to the scarcity of scientific research on pain relief that involves sheep as an animal model in experimental studies, researchers frequently face challenges in determining appropriate analgesic protocols for these animals (Stillman & Whittaker, 2019). The discussion will center on commonly used anesthetic agents, including combinations of ketamine, midazolam, and butorphanol, which have been shown to be effective in minimizing stress and physiological disturbances in sheep.

This article provides detailed steps and measures for preanesthetic protocols to ensure that sheep undergo a safe and effective anesthesia experience, ensuring optimal pain management during experimental surgical procedures.

MATERIALS AND METHODS

The information presented in this article is based on scientific data obtained from the evaluation of 13 general anesthetic protocols. The research was carried out at the University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, in compliance with the ethical guidelines for animal welfare, with all necessary permits and approvals obtained.

Thirteen adult sheep (*Ovis aries*) of different breed, aged 2-4 years, weighing 43-67 kg (with a mean body weight of 54.11 kg) were anesthetized at the Faculty of Veterinary Medicine of Bucharest, for different experimental surgical procedures.

The premedication intramuscularly (i.m.) protocol consisted of midazolam (0.2 mg/kg, i.m.), ketamine (5 mg/kg, i.m.), and butorphanol (0.1 mg/kg, i.m.) and fallowed by intravenous (i.v.) induction with propofol (3-5 mg/kg, i.v.) or alfaxalone (1-3 mg/kg, i.v.), intubation and maintenance with isoflurane (1.0-3.0 vol %) in oxygen 100%.

RESULTS AND DISCUSSIONS

Before anesthesia, it is essential to acknowledge that sheep are sensitive to stress, making the implementation of appropriate handling techniques crucial for their welfare. Inadequate or inappropriate physiological, immunological, metabolic, and behavioral responses can result in stress, leading to distress characterized by poor health or compromised well-being (Ignătescu et al., 2018), which may ultimately affect the outcomes of the procedure or the research.

Sheep (*Ovis aries*) are ruminants with a complex stomach system consisting of four compartments: the rumen, reticulum, omasum, and abomasum. While this system aids in digesting fibrous plant material, it also poses specific risks during general anesthesia. The primary concerns are regurgitation and aspiration of ruminal contents. Also, sheep can produce an increased saliva volume when under anesthesia, leading to airway obstruction. Recognizing these risks and applying preventive measures is crucial for safeguarding the animals' well-being and ensuring the success of the experimental procedures (Costea et al., 2022).

The choice of anesthesia and analgesia protocols was based on species-specific characteristics, the type of surgery, anticipated pain, and the available equipment. After analyzing these parameters, we decided to implement a 7-step preanesthetic protocol, including fasting management, preanesthetic evaluation, selection of preanesthetic medication, preanesthetic monitoring, administration of premedication, sheep stabilization before anesthesia induction, and post-preanesthetic monitoring.

The first step of our study was fasting management. Fasting in sheep it is necessary to reduce the risk of regurgitation during general anesthesia. Fasting has a limited effect on the volume of contents in the rumen but may reduce the incidence of ruminal tympany, which is gas accumulation in the rumen caused by bacterial fermentation (Wang et al., 2023). To avoid complications the sheep in our study were not given food 24 hours before general anesthesia and had unrestricted access to water until the premedication protocol was administered.

After the fasting period, the second step was to conduct a preanesthetic evaluation of the sheep. This was done in a low-stress environment, avoiding harsh restraint methods and loud noises to minimize stress. The preanesthetic evaluation included a clinical physical examination and the measurement of the sheep's weight.

We conducted for all the cases a comprehensive physical examination to evaluate the overall health of the sheep before administering anesthesia. This examination focused on the respiratory, cardiovascular, and digestive systems. We monitored the respiratory rate to identify any pre-existing respiratory conditions that could complicate the anesthesia process. Evaluating heart rate and rhythm was equally important, as any irregularities could affect the animal's response to anesthetic agents. Additionally, we checked the gastrointestinal tract of

the sheep for signs of distension or discomfort, which could indicate digestive issues requiring special consideration during surgery.

Hematological and biochemical tests were conducted to evaluate the clinical and metabolic status of the sheep (Mihai et al., 2023).

After the evaluation, the entire group of 13 sheep was classified as the ASA 1-2 risk group, according to the American Society of Anesthesiologists veterinary medicine standards, indicating a normal, healthy patient without any organic disease.

Sheep's body weight was accurately measured, which is crucial for determining the correct dosages of anesthetic agents. Since anesthetic drugs are typically administered based on weight, any inaccuracies could lead to underdosing or overdosing, both of which pose significant risks. An electronic scale was used to obtain precise weight readings, and excluded the weight of the wool to obtain precise weight estimation. A "wool-free weight" formula (Costea et al., 2022) was applied for the entire group by reducing the measured total body weight by 5%.

In the third step of our research, we focused on selecting preanesthetic medication. Preanesthetic drugs have several important functions, including reducing the amount of anesthetic needed for induction and maintenance, calming the animal for a smoother induction, and contributing to a more stable anesthesia. The choice of premedication is based on the results according to the preanesthetic examination and on the expected maintenance time of anesthesia.

Sedatives, sedative-opioid combinations, and anesthetic agents like ketamine can be used to achieve the desired sedation effects (Hall et al., 2001).

The preferred routes of administration, known for providing more predictable outcomes, are intramuscular or intravenous.

The selected cases received premedication protocols using benzodiazepines as the sedative of choice for sheep, typically in combination with other agents like opioids, ketamine.

The intravenous administration of a benzodiazepine, such as midazolam at a dose of 0.10-0.25 mg/kg, can produce sedation and ataxia, typically lasting for about 30 minutes. When administered intramuscularly at the same dose range (0.10-0.25 mg/kg), midazolam significantly increases the depth of sedation, especially when combined with another sedative or opioid. For instance, butorphanol at 0.1 mg/kg has a rapid onset of action and, when given 5-10 minutes before midazolam or diazepam, and ketamine, helps facilitate a smooth and relaxed induction of anesthesia. The effect typically lasts for 1-2 hours (Hall et al., 2001). The combination of ketamine, a benzodiazepine (midazolam), and an opioid (butorphanol) provides moderate sedation, and effective analgesia, and has minimal impact on cardiovascular function (White & Taylor, 2000).

α2-agonists may be less effective for pain relief in sheep compared to other animals, and at higher doses, they can cause excitement or changes in behavior (Kästner, 2006). Xylazine is a popular choice, but it can reduce ruminal motility and lead to bloat and is likely to develop pulmonary edema (Bacon et al., 1998). Ketamine can be administered alongside xylazine and butorphanol to achieve sedation and pain relief.

All these premedication combinations allow for administering smaller doses of each drug, thereby reducing potential side effects and facilitating a multimodal approach.

Based on these considerations, we analyzed the best combination of sedatives and pain relievers to keep the sheep calm during surgery and manage their pain effectively. By carefully selecting our preanesthetic medication, we aimed to optimize both sedation and pain relief, thereby improving the overall safety and effectiveness of the surgical procedures.

We decided to administer the premedication via intramuscular injection into the triceps muscle. After choosing the preanesthetic medication, we conducted the preanesthetic monitoring to ensure the safety and stability of the sheep. Before administering any anesthetic drugs, we recorded the baseline heart and respiratory rates for each sheep. This was crucial to establish a reference point, allowing us to identify any potential abnormalities or changes in the sheep's condition after sedation and during the anesthetic process.

Body temperature and pulse oximetry were monitored to ensure the sheep were in optimal

condition for anesthesia and surgery. It is crucial to maintain stable body temperature, as hypothermia can complicate the anesthetic process. Continuous monitoring of pulse oximetry was vital for assessing oxygenation and ventilation after administering the preanesthetic medications (Figure 1).



Figure 1. Monitoring during anesthesia (original)

To address the significant saliva production of approximately 6-16 liters over 24 hours and to prevent regurgitation, the sheep were positioned in sternal recumbency with their heads elevated (Somers, 1957).

Tear production decreases during anesthesia in sheep, making it essential to implement protective measures to prevent eye dryness and potential lesions. Proper eye care, such as closing and taping the eyes or applying appropriate lubricants, is crucial for maintaining ocular health during anesthesia. Ideally, protective eye care measures should be implemented starting from the premedication phase (Tudor et al., 2018). Early intervention ensures optimal eye protection throughout the entire anesthetic process, reducing the risk of ocular complications in sheep (Pavel et al., 2024). After administering the premedication, a 20-gauge intravenous catheter was easily inserted into the cephalic vein to prepare for the induction of anesthesia (Figure 2).

The venous catheter helps administer intravenous fluids, additional doses of parenteral anesthetic and analgesia, and emergency medication if needed. Before inducing general anesthesia, it was mandatory to stabilize each animal to ensure the best possible conditions for the procedure. This step was the sixth phase of our preanesthetic protocol.



Figure 2. Intravenous catheter into the cephalic vein (original)

The main aim was to ensure that the sheep were calm and stable before anesthesia. To achieve this, we used various techniques to minimize stress, such as gentle handling and maintaining a quiet environment. By creating a low-stress atmosphere, we aimed to help the sheep feel secure, which is crucial for their safety and a smooth transition into anesthesia.

Another important goal was to support the cardiovascular function and to maintain the blood pressure of the sheep. We achieved this by administering intravenous fluids based on the specific condition, hydration status, and cardiovascular health of each animal (Musk, 2024).

During anesthesia sheep received a maintenance rate of fluids of 5 ml/kg/h Ringer's Lactate solution. This step was crucial for ensuring adequate hydration and promoting hemodynamic stability, thereby enhancing the overall safety of the surgical procedures.

After administering preanesthetic medications a thorough monitoring protocol was implemented to ensure safety and stability during the transition into anesthesia. We were prepared to reverse sedation or address any potential side effects that could arise during this phase. If excessive sedation or respiratory depression occurred, we were ready to modify our approach and provide necessary supportive care. This allowed us to manage any potential complications promptly and effectively. Through this anesthetic monitoring, we prioritized the well-being of each sheep, ensuring their stability throughout the induction process, which ultimately contributed to the success of the surgery procedures.

CONCLUSIONS

Adherence to comprehensive preanesthetic protocols is crucial to minimizing the risks of regurgitation and aspiration of ruminal contents in sheep (*Ovis aries*). Under conditions where a rigorous preanesthetic evaluation protocol is followed and preparations are made for potential risks and complications, sheep can serve as a valuable resource for research, yielding predictable results.

This includes careful management of fasting, the administration of appropriate medications, the use of specific preanesthetic techniques, and continuous monitoring. By following these seven steps, we can greatly reduce potential risks and enhance the safety and well-being of sheep undergoing general anesthesia.

This approach is vital for ensuring successful outcomes in experimental surgical procedures involving sheep. For researchers and veterinarians involved in such interventions, this article provides a comprehensive guide to the essential steps of the preanesthetic process, offering guidelines that can enhance success rates and reduce potential complications.

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CHALLENGES AND OPPORTUNITIES IN THE APPLICATION OF ARTIFICIAL INSEMINATION IN SHEEP BREEDING

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Abstract

The use of artificial insemination (AI) in sheep breeding poses both considerable obstacles and exciting prospects. This research delves into the complexities of artificial insemination, focussing on factors influencing fertility rates, semen handling techniques, and the effect of hormone treatments on reproductive success. Several studies reveal that the success of AI is dependent on ideal sperm quality, which can be negatively impacted by cryopreservation procedures; for example, the concentration of sperm upon freezing has been found to influence post-thaw quality and subsequent reproductive outcomes in sheep. Furthermore, the method of insemination used - whether cervical or laparoscopic - is important, with laparoscopic techniques frequently providing greater pregnancy rates due to their ability to avoid anatomical challenges presented by the ewe's cervix. Furthermore, synchronising oestrus with hormonal therapies, such as oxytocin or equine chorionic gonadotropin (eCG), has been found as a critical element in improving the timing and efficiency of AI. However, the unpredictability in conception rates is still a problem, with reported statistics ranging from 20% to 70% depending on the procedures used and the settings under which AI is conducted. Environmental influences, such as temperature and stress, impair reproductive results, especially in the vital early phases of embryo development. The aim of this review is to summarise current research in order to identify best practices and new opportunities for improving the efficacy of AI in sheep breeding.

Key words*: artificial insemination, Fertility Rates, sheep breeding.*

INTRODUCTION

Artificial insemination (AI) is an important reproductive tool in livestock breeding, enabling the genetic enhancement of multiple species, including sheep. The technique entails the deliberate introduction of sperm into a female's reproductive tract to facilitate conception without natural mating. AI has transformed livestock breeding by facilitating the swift distribution of better genetics, therefore improving production and genetic diversity in herds. The efficacy of artificial insemination is dependent on various parameters, including semen quality, the time of insemination in relation to the female's oestrus cycle, and the skill level of the inseminator (Paul, 2024; Sitepu et al., 2023; Faigl et al., 2012). Artificial insemination is pivotal in sheep breeding for genetic advancement and productivity advancement. The use of AI facilitates use of genetically superior rams, hence significantly enhancing the genetic quality of the offspring. This is particularly important in ovine husbandry, where conventional breeding techniques may yield restricted genetic diversity and diminished genetic advancement. Through the application of AI, breeders can more swiftly include desirable traits, including enhanced wool quality, disease resistance, and superior reproductive performance (Sitepu et al., 2023; Faigl et al., 2012). Additionally, AI can mitigate inbreeding concerns by allowing the introduction of genetic material from distant populations, hence enhancing the overall

genetic diversity (Khandoker, 2023; Putri et al., 2021). Research indicates that pregnancy rates linked to artificial insemination in sheep may fluctuate, although under optimal settings, they can attain considerable levels. Artificial insemination pregnancy rates in sheep can vary from 26% to 61%, influenced by factors including the timing of insemination and the quality of the frozen semen utilised (Sitepu et al., 2023; Jha et al., 2020). The adoption of fixed-time artificial insemination (FTAI) procedures has improved the efficacy of artificial insemination by synchronising oestrus in ewes, facilitating more accurate insemination timing and potentially elevating conception rates (Negreiros et al., 2020; McCappin & Murray, 2011). The economic ramifications of AI in sheep breeding are significant. The cost-effectiveness of artificial insemination relative to natural mating is significant, as it diminishes the necessity for maintaining numerous rams and facilitates the utilisation of superior frozen semen from elite rams, which can be transported over extensive distances without compromising viability (Asaduzzaman et al., 2021). This not only reduces operational expenses but also allows farmers to obtain superior genetics that would otherwise be inaccessible in their local breeding populations (Paul, 2024; Asaduzzaman et al., 2021; Siti & Rachmawati, 2024; Faigl et al., 2012). Besides genetic and economic advantages, AI also enhances the health and welfare of sheep populations. AI diminishes the necessity for physical interaction among breeding animals, hence mitigating the possibility of disease transmission, a critical consideration in densely populated agricultural settings (Morrell et al., 2022; Faigl et al., 2012). Moreover, the capacity to select for particular features via AI can result in healthier and more resilient sheep, capable of thriving in various environmental conditions (Paul, 2024; Putri et al., 2021; Sarini et al., 2023; Musriati et al., 2024; Sirajuddin et al., 2018). The implementation of modern technology, including laparoscopic insemination procedures, has been proven to further improve conception rates, underscoring the necessity for constant improvement and adaptability in AI methodologies (McCappin & Murray, 2011; Faigl et al., 2012).

The aim of this review is to analyse the challenges and opportunities associated with artificial insemination in sheep breeding by evaluating factors affecting fertility, semen handling techniques, insemination methods, and hormonal synchronization.

MATERIALS AND METHODS

The materials for this review were collected through a systematic search of academic databases such as PubMed, ScienceDirect, and Google Scholar, using key words such as "artificial insemination in sheep", "semen handling techniques", and "fertility factors in ovine reproduction". The selected papers were evaluated based on their relevance, publication date, and methodology and the retrieved data were compared to highlight the field's challenges and potential.

RESULTS AND DISCUSSIONS

Factors influencing AI success in sheep

Semen quality and handling techniques

Sperm quality is a crucial factor influencing reproduction results in various species, including livestock and humans. High-quality sperm, defined by superior motility, morphology, and membrane integrity, is positively associated with successful fertilisation rates (Zeng et al., 2021; Elmagd et al., 2022). Research indicates that sperm motility is a crucial predictor of fertility, with enhanced mass sperm motility correlating with elevated fertility rates in sheep (David et al., 2015). The integrity of DNA is crucial, since fragmentation can negatively impact fertilisation potential and subsequent embryonic development (González-Marín et al., 2012). Cryopreservation substantially affects sperm viability, frequently resulting in diminished motility and vitality after thawing. The freezing process can cause oxidative stress and lipid peroxidation, undermining sperm membrane integrity and functionality (Perumal et al., 2010; Jiménez-Rabadán et al., 2016). Studies demonstrate that including antioxidants in the cryopreservation process can alleviate adverse effects, hence improving post-thaw sperm quality (Perumal et al., 2010; Perumal et al., 2013). The concentration of sperm during

freezing is critical; elevated concentrations can increase the probability of preserving viable sperm after thawing, thereby improving reproductive results (Madeddu et al., 2024).

Insemination methods

Cervical and laparoscopic inseminations are two common techniques for artificial insemination (AI) in sheep, each possessing unique benefits and drawbacks. Cervical insemination (CAI) is less intrusive and more economical, making it accessible to numerous breeders. The anatomical limitations of the ewe's cervix, marked by its small and convoluted configuration, frequently limit efficient semen deposition, resulting in reduced conception rates, generally under 30% with frozen-thawed semen (Richardson et al., 2019; Álvarez et al., 2019). Laparoscopic insemination (LAI) facilitates direct intrauterine semen deposition, circumventing cervical barriers, which may significantly enhance fertility outcomes, especially with suboptimal sperm quality (El-Badry, 2014; Sathe, 2018). Research indicates that LAI can attain conception rates over 70% under ideal circumstances (Masoudi et al., 2017; McCappin & Murray, 2011). Various strategies can be utilised to enhance insemination efficiency. Synchronisation methods, including progesterone-CIDR, can optimise ovulation time and augment conception rates (Moreira et al., 2023; Álvarez et al., 2019). Furthermore, maintaining high-quality sperm via appropriate cryopreservation methods and reducing oxidative stress during storage are essential for optimising post-thaw viability (Bülbül et al., 2024). Moreover, innovations in transcervical methods, including the surgical excision of cervical folds, could enhance accessibility and elevate pregnancy rates (Pau et al., 2020). Although LAI presents enhanced fertility potential, the decision between CAI and LAI must take into account aspects such as expense, necessary skills, and particular breeding objectives.

Hormonal synchronization and fertility regulation

Hormonal therapies, such equine chorionic gonadotropin (eCG) and oxytocin, are crucial for oestrus synchronisation in sheep. eCG is frequently employed to promote follicular maturation and induce oestrus, whilst oxytocin can aid in uterine contractions and boost reproductive results after insemination (Silva et al., 2020; Souza et al., 2021). The efficacy of these hormonal treatments can result in synchronised oestrus, facilitating enhanced breeding control and elevated lambing rates (Souza et al., 2021). The influence of hormone regimens on reproductive outcomes is significant. Research indicates that the administration of eCG alongside progestogens can markedly enhance the proportion of ewes displaying oestrus and increase the conception rates (Mohan, 2023; Quintero-Elisea et al., 2011). Hormonal therapies can prolong and intensify oestrus, which are essential for successful mating (Mohan, 2023). Nonetheless, fluctuations in conception rates may arise depending on the synchronisation techniques utilised. Research demonstrates that certain treatments attain high synchronisation rates (up to 100%), whilst others may produce lower rates influenced by factors such as individual animal responses, ambient conditions, and nutritional status (Souza et al., 2021; Wu et al., 2022).

Challenges and future directions

Regarding challenges, heat stress significantly impacts fertility and embryonic development in livestock, especially in sheep and cattle. Increased temperatures can negatively impact oocyte maturation, fertilisation, and subsequent embryonic development. Research indicates that exposure to temperatures between 39.5-41.0°C during the initial phases of in vitro culture (IVC) can diminish the growth rates of bovine embryos, resulting in decreased blastocyst formation rates (Alves et al., 2013; Namekawa et al., 2010). Moreover, heat stress can interfere with follicular dynamics, leading to a decrease in viable follicles and diminished reproductive success (Ratchamak et al., 2022; Ratchamak et al., 2021).

Proper nutrition reduces certain adverse effects of heat stress by enhancing metabolic processes and preserving overall health (Feugere et al., 2022; Ortega et al., 2016). Effective management strategies, including the provision of shade, sufficient water, and nutritional supplements, can bolster resilience to heat

stress and promote reproduction rates (Ratchamak et al., 2021; Crews et al., 2015). Implementing stress reduction measures is crucial for enhancing artificial insemination (AI) results. Methods include environmental changes dietary interventions, and the administrations of stress-relieving supplements such as melatonin have demonstrated potential in enhancing reproductive efficiency (Ratchamak et al., 2022; Ortega et al., 2016). Producers can improve the reproductive performance of their flocks by addressing environmental and dietary issues, especially during heat stress periods.

The future of artificial insemination (AI) in sheep is set for substantial progress, especially in semen preservation methods, laparoscopic technology, genetic selection, and comprehensive optimisation methodologies. Innovations in cryopreservation procedures, such as the use of novel cryoprotectants and antioxidants, are essential for improving the viability of frozen-thawed semen. Recent studies indicate that the use of antioxidants during freezing can reduce oxidative stress, enhancing post-thaw motility and fertility rates (Pelayo et al., 2019; Yang, 2024). The investigation of different cryoprotectants may improve sperm viability during freezing and thawing, resulting in superior outcomes in artificial insemination programs (Akter et al., 2020).

Laparoscopic AI has already exhibited higher conception rates compared to conventional cervical insemination. Future advancements may concentrate on improving laparoscopic procedures to diminish invasiveness and facilitate use, possibly integrating real-time imaging technology to augment precision during insemination (Akter et al., 2020). Investigating the optimisation of insemination timing in relation to ovulation is essential, especially when utilising frozen-thawed semen, which has a restricted viability period (Akter et al., 2020). The genetic selection of traits that improve compatibility with AI is an increasing area of interest. Identifying and choosing genetic markers linked to reproductive efficiency, semen quality, and fertility can substantially enhance flock productivity (Pelayo et al., 2019; Yang, 2024; Nosrati et al., 2018; Gheorghe-Irimia, 2023). Genome-wide association studies (GWAS) have identified candidate genes associated with reproductive traits, which can guide breeding programs to improve artificial insemination success rates (Yang, 2024; Nosrati et al., 2018). Ongoing research is crucial for enhancing AI procedures. This involves examining the impact of environmental variables, such as thermal stress, on reproductive efficacy and assessing nutritional approaches that may improve fertility during artificial insemination (Narayan et al., 2018; Boareki et al., 2021; Gheorghe-Irimia, 2024; Șonea et al., 2023a; Șonea et al., 2023b). Furthermore, the combination of genomic selection with conventional breeding methodologies might accelerate genetic advancements and enhance the general resilience of sheep populations (Zhang et al., 2023; Li et al., 2020).

CONCLUSIONS

Artificial insemination (AI) has significantly progressed sheep breeding by facilitating genetic enhancement, boosting productivity, and augmenting genetic diversity. Despite all of its advantages, AI encounters significant obstacles, including fluctuations in conception rates, deterioration of semen quality owing to cryopreservation, and the complicated nature of insemination methodologies. The implementation of hormonal synchronisation and enhanced insemination techniques, especially laparoscopic artificial insemination, has facilitated the optimisation of conception rates; yet environmental and management aspects remain pivotal to reproductive success.

Continuous research is essential for improving semen preservation methods, advancing artificial insemination procedures, and discovering genetic markers linked to reproductive effectiveness. Moreover, mitigating environmental stresses and optimising nutritional management can further improve AI results in sheep breeding. Through the integration of technical improvements and optimal management techniques, AI can remain a crucial instrument for sustainable and effective sheep reproduction, guaranteeing economic viability and genetic advancement within the industry.

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ADVANCING SHEEP REPRODUCTION: THE SCIENCE AND PRACTICE OF LAMBING INTENSIFICATION

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Abstract

The physiological basis for lambing intensification is rooted in the reproductive physiology of ewes, which governs lambing intervals and is influenced by factors such as photoperiod and seasonal breeding patterns. Strategies for intensification include the implementation of accelerated lambing systems, which utilize hormonal treatments and photoperiod manipulation to achieve three lambings within two years. Genetic selection for prolific breeds, such as Finnsheep and Romanov, is emphasized as a critical component of breeding programs aimed at enhancing lamb output. Nutritional management strategies, including flushing and balanced diets during gestation and lactation are also discussed as vital for improving reproductive efficiency. Furthermore, the application of reproductive technologies such as artificial insemination, embryo transfer, and oestrus synchronization techniques is highlighted to achieve tighter lambing windows and increased reproductive rates. Despite the potential benefits of lambing intensification, several challenges and limitations must be addressed. Health and welfare concerns arise from the increased metabolic demands placed on ewes, leading to risks of reproductive exhaustion and poor maternal care. Additionally, the management of diseases associated with higher lambing frequencies, such as mastitis, is critical. Economic constraints, including increased labour demands and costs associated with feed and veterinary care, pose further challenges to the adoption of intensive systems. In conclusion, this paper aims to elucidate the intricate interplay between physiological mechanisms, management strategies, and the challenges faced in advancing lambing intensification, ultimately contributing to the sustainability and productivity of sheep farming systems.

Key words*: ewe management, prolificacy traits, reproductive physiology.*

INTRODUCTION

Lambing intensification is a vital component of contemporary sheep husbandry that seeks to improve productivity and efficiency in lamb production. This method involves increasing the frequency of lambing cycles, optimising breeding strategies, and enhancing management practices in order to achieve higher lamb survival rates and superior meat quality. The importance of lambing intensification can be understood through its effects on productivity, economic viability, and sustainability in the sheep farming industry. The main advantage of lambing intensification is the increase of production. Research indicates that the use of an accelerated lambing system can enhance lambing percentages and decrease lambing intervals, therefore optimising the yield from each breeding ewe (Fogarty & Mulholland, 2012; Fozi et al., 2020). This is particularly important in areas where the demand for premium lamb meat is rising, prompting producers to enhance their production techniques to satisfy consumer desires for fine and superfine wool as well as high-quality meat (Krehbiel, 2013; Knapik et al., 2017). Furthermore, the intensification of lambing enhances the economic sustainability of sheep husbandry. Increasing lamb production enables

farmers to enhance economic stability and mitigate financial risks linked to livestock husbandry. The capacity to generate additional lambs during the same period facilitates improved cash flow management and may result in increased profitability, particularly in competitive markets (Benoît & Laignel, 2014). Moreover, the implementation of advanced breeding and management techniques, including hormonal manipulation for oestrous cycle regulation, can significantly improve lambing efficiency, but these approaches may not consistently adhere to conventional extensive management systems (Fozi et al., 2020). Sustainability represents a vital element of lambing intensification. Studies indicate that more intensive lamb production systems can decrease greenhouse gas emissions per kilogramme of meat produced, mainly due to enhanced feed efficiency and superior management of grazing grounds (Gill et al., 2010; Geß & Hazar, 2023). Through the optimisation of production processes, sheep producers can enhance environmental sustainability while ensuring economic viability. Moreover, the incorporation of regenerative agriculture strategies, including biosequestration and enhanced soil management, can further alleviate the negative environmental impacts of increased lamb production.

The aim of this review is to explore the physiological mechanisms, management strategies, and challenges associated with lambing intensification, with the goal of enhancing reproductive efficiency and sustainability in sheep farming systems.

MATERIALS AND METHODS

The data for this review was collected through a thorough literature search in academic databases such as PubMed, ScienceDirect, Scopus, and Google Scholar, using key words like "lambing intensification", "accelerated lambing cycles", "reproductive efficiency in sheep", and "sustainable sheep farming". Relevant peer-reviewed articles, conference proceedings, and industry reports were selected for their scientific credibility, publication date, and methodological rigour, and the extracted data was systematically analysed.

RESULTS AND DISCUSSIONS

Physiological basis of lambing intensification

Environmental factors, especially photoperiod, have a significant impact on ewes' seasonal breeding patterns and are closely linked to their reproductive physiology. It is crucial to comprehend how ewes' hormones control their reproductive cycles in order to maximise breeding strategies in sheep farming. Ewes display a seasonal breeding pattern, mostly determined by the length of daylight they receive throughout the year. The hypothalamic-pituitary-gonadal (HPG) axis controls this seasonal reproductive behaviour, which is defined by a separate breeding season that usually takes place in the autumn. Gonadotropin-releasing hormone (GnRH), which triggers the pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH), resulting in the development of ovarian follicles and ovulation, is secreted by the HPG axis (Chemineau et al., 2004; Narayan et al., 2018; Dupré et al., 2010). Photoperiod has a vital function in controlling the reproductive cycles of sheep. Melatonin, a hormone secreted by the pineal gland and essential for indicating ewes' reproductive state, is influenced by the duration of sunshine. During longer days, melatonin secretion decreases, which promote reproductive activity, while shorter days increase melatonin levels, were inhibiting reproductive functions (He et al., 2023; Wagner et al., 2007). According to studies, ewes kept on long-day photoperiods perform better reproductively, exhibiting higher ovulation rates and an earlier onset of oestrus (Dardente & Lomet, 2018; Gómez-Brunet et al., 2008). Furthermore, research has shown that variations in photoperiod affect the expression of particular genes in the pituitary and hypothalamus. For instance, lengthy photoperiods are associated with reproductive activation and a substantial increase in the expression of RFamide-related peptide (RFRP) (Dardente et al., 2008). Furthermore, the thyroid hormone system also interacts with photoperiod cues to regulate reproductive cycles, indicating a complex interplay between environmental factors and hormonal responses (Dardente & Lomet, 2018; Wang et al., 2022). The hormonal regulation of reproductive cycles

in ewes is primarily governed by the interactions of GnRH, LH, FSH, and sex steroids such as estrogen and progesterone. This hormonal balance can be upset by stressors such as physiological stress and environmental changes, which can impact reproductive performance (Narayan et al., 2018; Dardente et al., 2022). For instance, elevated glucocorticoid levels due to stress can negatively impact the secretion of reproductive hormones, thereby affecting ovulation and fertility (Narayan et al., 2018; Dardente et al., 2022). Moreover, the role of leptin, a hormone associated with energy balance, has been highlighted in the context of reproductive physiology. Leptin levels can modify the sensitivity of the hypothalamus to GnRH, consequently changing the timing and effectiveness of reproductive cycles (Zieba et al., 2019; Biernat et al., 2021). For ewes to be in the best possible condition for breeding, metabolic signals and reproductive hormones must be integrated, especially during seasonal contexts when energy availability may vary (Archer et al., 2005; Dobbins et al., 2004).

Strategies for lambing intensification

Accelerated lambing systems are increasingly being adopted in sheep farming to enhance productivity by allowing ewes to lamb more frequently within a given timeframe. This method can greatly enhance the number of lambs produced per ewe and boost overall farm profitability because it usually aims for three lambings in two years. Hormonal therapies, photoperiod adjustment, genetic selection for prolific breeds, and nutritional management are some of the crucial elements involved in the implementation of such systems. The idea of having three lambings in two years stems from the desire to increase ewes' capacity for reproduction. To enable more frequent lambing, this approach necessitates the careful control of breeding cycles and the application of hormonal treatments to synchronise ovulation and oestrus (Naqvi et al., 2011; Cirne et al., 2016). Farmers may successfully raise lambing rates by using methods like controlled breeding and artificial insemination, which is essential for satisfying consumer demand for lamb meat and wool. In order to increase reproductive efficiency, ewes are frequently given hormonal therapies, such as gonadotropins and prostaglandins, to promote oestrus and ovulation (Naqvi et al., 2011; Farrag, 2019). Furthermore, ewes' reproductive cycles can be significantly regulated by photoperiod alteration. Farmers can further promote the goal of rapid lambing by manipulating light exposure to mimic seasonal variations that encourage ewes to breed outside of their natural season. Improved reproductive performance and a higher frequency of lambing can result from this combination of environmental and hormonal control techniques. Another essential element of faster lambing systems is genetic selection. Breeds like Romanov and Finnsheep are frequently used in breeding projects to increase lamb production because of their high prolificacy (Nosrati et al., 2018; Sarvinda et al., 2022). Compared to other breeds, Romanov ewes usually have bigger litter numbers, and Finnsheep are renowned for their capacity to give birth to several lambs throughout a pregnancy. Sheep flocks' total reproductive capacity can be greatly enhanced by including these prolific breeds into breeding programs. The effectiveness of rapid lambing systems depends on efficient breeding programs that emphasise genetic enhancement techniques. In order to find and spread desirable features linked to reproductive performance, these algorithms frequently employ genomic selection techniques (Yang et al., 2024; Gizaw et al., 2011). Farmers can improve the genetic potential of their flocks and raise lamb production and economic results by selecting for qualities like litter size and ovulation rate. An essential component of assisting rapid lambing systems is nutritional control. It has been demonstrated that feeding sheep a high-energy diet before breeding, known as flushing, improves body condition and increases ovulation rates, which in turn improves reproductive success (Khotijah et al., 2022; Naqvi et al., 2011; Farrag, 2019). Studies show that ewes fed flushing diets have better lambing results and higher reproductive rates, especially when paired with hormonal therapies (Cirne et al., 2016; Farrag, 2019). Furthermore, ewes and their lambs' health and production depend on maintaining appropriate meals during pregnancy and lactation. Successful lamb

rearing depends on ewes receiving adequate nourishment during these crucial times, which also supports foetal growth, improves milk production, and enhances ewes' general wellbeing (Sejian et al., 2010).

Reproductive technologies in lambing intensification

Key technologies include artificial insemination (AI), embryo transfer (ET), and oestrus synchronization techniques. These techniques promote more effective breeding techniques and enhance genetic diversity, which eventually results in higher lamb production. A popular reproductive technique in sheep farming is artificial insemination, which eliminates with the necessity for natural mating and makes use of superior genetics. AI can boost genetic advancement in flocks by dramatically increasing the number of ewes bred with superior semen from chosen rams (Gibbons et al., 2019). The time of insemination in relation to ovulation, the quality of the semen used (fresh or frozen), and the insemination technique utilised are some of the aspects that affect the success of AI (Saha et al., 2021; Sitepu et al., 2023). Although AI can be useful, there is still opportunity for improvement in its use, as studies have shown that conception rates with AI in sheep range from 26% to 61% (Sitepu et al., 2023). Another cutting-edge reproductive technique that enables the quick reproduction of animals with improved genetic makeup is embryo transfer. According to Khan et al. (2022), this method entails harvesting embryos from donor ewes that have experienced superovulation and then transferring them to recipient ewes. Because ET enables the birth of numerous offspring from a single donor ewe in a single breeding season, ET can greatly increase the reproductive output of high-performing ewes. According to research, the quality of the embryos and the time of the embryo transfer can have an impact on the pregnancy rates linked to ET (Bergstein-Galan et al., 2018; Romão et al., 2016). Techniques for oestrus synchronisation are crucial for maximising AI and ET time. These techniques enable more effective breeding by using hormone treatments to cause synchronised oestrus in a group of ewes (Berean et al., 2021). There are several methods that can successfully synchronise ovulation and enhance ewes' overall reproductive performance, such as the use of progestagens, gonadotropins, and melatonin implants (Lu et al., 2021; Korkmaz & Yaprak, 2022). It has been demonstrated that synchronisation methods shorten the lambing window, enabling more focused lambing times and improving lambing and post-lamb care management (Zhang et al., 2024). Reproductive rates and lambing windows are significantly impacted by the combination of AI, ET, and oestrus synchronisation approaches. Sheep breeders can decrease the interval between lambing events and increase lambing rates by utilising these technologies. For example, fixed-time artificial insemination (FTAI) streamlines the breeding procedure and increases the efficiency of lamb production by enabling insemination without the necessity for oestrus identification (Lu et al., 2021). According to studies, using synchronisation techniques can result in a more consistent lamb crop and higher lambing percentages, both of which are advantageous for management and marketing (Khan et al., 2022).

Challenges and limitations

A number of challenges and restrictions exist when rapid lambing technologies are used in sheep farming, which may have an effect on the sustainability, economic viability, and well-being of ewes. For such systems to be successful in the future, these issues must be addressed. Reproductive exhaustion is one of the main health and welfare issues related to lambing intensification methods. Frequent lambing cycles can cause ewes to undergo more physiological stress, which can result in problems like decreased fertility, longer recovery periods, and lower maternal care (Lomet et al., 2020). Frequent breeding needs may lead to insufficient mother care, which is essential for lambs' survival and development. According to research, ewes that don't recover well between lambing episodes can provide less milk and behave worse as mothers, which would ultimately reduce the survival rate of the lambs (Thompson et al., 2011). Another major issue with increased lambing systems is disease management. Both ewe health and lamb growth may be negatively impacted by infectious

diseases like mastitis, which can spread more easily during concentrated lambing times because of the higher density of ewes and lambs (Skipor et al., 2021). The financial sustainability of sheep businesses can be further strained by mastitis, in particular, which can result in lower milk output and higher veterinary expenses (Fozi et al., 2020). To reduce these risks, effective disease management techniques - such as immunisation and biosecurity measures - are crucial. Accelerated lambing systems have a variety of economic effects. Because intense management during lambing periods might put a strain on existing labour resources, increased labour demands are a major concern (Herman et al., 2010). The additional workload brought on by frequent lambing may make it difficult for farmers to locate enough workers, which could raise operating expenses (Gheorghe-Irimia et al., 2023). The nutritional needs of ewes during pregnancy and lactation, as well as the necessity of routine veterinary interventions to address health issues resulting from intensified breeding practices, can also drive up the costs of feed and veterinary care (Lacasse et al., 2014). Furthermore, changes in the market and the demand for lamb products have an impact on the financial viability of accelerated lambing systems. Higher lamb production can boost profits, but it also puts farmers at risk in the market, especially if supply outpaces demand (Abecia et al., 2017). Therefore, to guarantee the sustainability of accelerated lambing methods, thorough financial planning and market analysis are crucial. When it comes to accelerated lambing techniques, sustainability is a crucial factor (Gheorghe-Irimia et al., 2024; Șonea et al., 2023a). To make sure that these solutions don't jeopardise the environment or the health of the flock, the long-term effects on ewe productivity and farm profitability must be assessed. Frequent lambing can cause physiological stress that eventually reduces ewe longevity and productivity, which will ultimately impact the farm's total profitability (Thompson et al., 2021). Furthermore, it is important to take into account how enhanced lambing procedures may affect the ecosystem. If improperly managed, increased feed needs and higher stocking densities might result in increased nutrient runoff and environmental degradation (Herman et al., 2017). While preserving productivity, sustainable techniques like integrated pest management and rotational grazing can help lessen these negative effects on the ecosystem.

CONCLUSIONS

Lambing intensification, particularly through accelerated lambing systems, relies on the strategic manipulation of reproductive physiology, including photoperiod responsiveness, exogenous hormone administration, and control of the oestrous cycle. The application of assisted reproductive technologies—such as oestrus synchronization, artificial insemination, and embryo transfer—permits tighter control over lambing intervals and facilitates genetic dissemination. Selection for highly prolific genotypes remains central to increasing ovulation rates and litter size, though this must be matched with appropriate nutritional regimens, such as flushing and optimized gestational feeding, to support fetal development and postnatal performance.

Despite the potential to significantly enhance reproductive output, intensification introduces physiological and metabolic challenges, including increased risk of reproductive fatigue, compromised maternal behavior, and elevated susceptibility to production-related diseases such as mastitis and ketosis. Furthermore, the biological limits of reproductive capacity, particularly in seasonal breeders, pose constraints on long-term system sustainability. The success of intensified systems is contingent upon precise reproductive monitoring, robust nutritional support, and health management protocols capable of mitigating negative outcomes. Future research should prioritize the development of ewe-centric management strategies that align endocrine manipulation with natural reproductive rhythms, explore genotype × environment interactions under intensive conditions, and evaluate the long-term effects of high-frequency lambing on ewe longevity, lamb viability, and overall system efficiency. Multidisciplinary approaches integrating physiology, genetics, nutrition, and health will be essential for optimizing reproductive

efficiency without compromising animal welfare or economic returns.

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PERFORMANCE EVALUATION OF 3-PART MULTISPECIES HEMATOLOGY ANALYZERS FOR WHITE BLOOD CELL COUNTING IN SHEEP

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Abstract

This study aimed to assess the accuracy and precision of 3-part multispecies hematology analyzers for white blood cell (WBC) counting in comparison to manual quantification in apparently healthy Sipli sheep (n = 60). Blood samples were collected once and analyzed using a hemocytometer with two different dilutions (1:20 and 1:40), referred to as WBC-1 and WBC-2, respectively. Automated WBC counting was performed using two multispecies veterinary hematology analyzers, WBC-R and WBC-B. The mean (± SE) values and reference intervals (RIs) for overall and group-wise data showed that only WBC-R fell within the physiological range for sheep, whereas WBC-1, WBC-2, and WBC-B reported lower values. A weak agreement was observed between the two multispecies analyzers, with a mean bias of -30.97 (upper limit: -14.56, lower limit: -46.77) and a standard deviation of bias of 8.37. The intraclass correlation coefficient (ICC) was also low (0.619), indicating poor consistency. Additionally, Lin's concordance correlation coefficient (LCCC) measured accuracy at 0.086, while precision was determined to be 0.603. In summary, hemocytometer-based manual WBC counting in sheep may lack accuracy. Among the tested analyzers, Rayto RT-7600Vet (China) produced WBC counts closest to the physiological range, making it a more suitable option for clinical use. It is concluded that 3-part hematology analyzers with predefined settings for sheep require calibration with separate set of RIs to ensure accurate analysis of sheep blood.

Key words*: hematological validations, multispecies hematology analyzers, point-of-care-tests.*

INTRODUCTION

The hemocytometer is widely regarded as the "standard manual tool" for blood cell counting. However, it has largely been replaced by highly sensitive and specific hematology analyzers, which offer rapid and automated cell counts along with a broader range of hematological parameters (Huang et al., 2023; Data Bridge Market Research, 2022). Despite these advancements, the hemocytometer remains a key reference tool for validating the accuracy of hematology analyzers.

Over the past two decades, significant achievements in hematology and improved the accessibility and precision of automated analyzers in both human and veterinary medicine (DeNicola, 2011). Sysmex Corporation (Japan) is recognized as a global leader in hematology analyzer development, while other countries, including China, the USA, France, and Germany, have also made substantial contributions to analyzer manufacturing. China, in particular, has been developing these analyzers for nearly 40 years, making it the second-largest market for global research and production (Song & Zhu, 2020; Absar et al., 2024). Since medical diagnoses rely heavily on analyzer-generated results, ensuring analytical validity through systematic verification is crucial before implementing a new hematology analyzer in clinical practice (Farooq et al., 2023a; Vis & Huisman, 2016).

In veterinary medicine, 3-part and 5-part multispecies hematology analyzers are extensively used for white blood cell (WBC) counting and differential leukocyte analysis as point-of-care tests (POCTs). The 5-part analyzers provide more detailed leukocyte differentiation, whereas 3-part analyzers

classify WBCs into three broad categories: lymphocytes, intermediate cells, and granulocytes (Daves et al., 2024; Farooq et al., 2025). This limited classification may compromise the accuracy of WBC counts, particularly in species with unique hematological characteristics, such as sheep. WBC counting in sheep poses additional challenges, especially when using 3-part multispecies hematology analyzers, which may not provide precise results. Previous studies suggest that WBC counts obtained from different analyzers can vary significantly, often producing erroneous values. In Pakistan, multispecies hematology analyzers are becoming increasingly popular for veterinary diagnostics, yet their accuracy, precision, and reliability for WBC counting in sheep have not been systematically evaluated. This study is the first of its kind to assess the accuracy and precision of 3-part multispecies hematology analyzers compared to manual quantification using a hemocytometer with two dilution methods in apparently healthy Sipli sheep.

MATERIALS AND METHODS

Geo-Location of the Study

The current research was carried out concurrently at two locations within The Islamia University of Bahawalpur (IUB), Pakistan: the Livestock Farm under the Faculty of Veterinary and Animal Sciences (FV & AS), and the Postgraduate Laboratory in the Department of Physiology. Both facilities are located near the outskirts of the Cholistan Desert - locally referred to as Rohi - in Bahawalpur, Punjab. The desert is an extension of the Great Indian Desert, which also encompasses the Rajhsatan Desert in India and the Thar Desert in Sindh Province, Pakistan. Distributed through an area of 26000 km², it begins around 30 km from the city of Bahawalpur, Punjab, Pakistan, and is situated between latitudes 27º42´and 29º45´North and longitudes 69º52´and 75º24´East. This region endures an arid subtropical continental climate with extreme temperatures, high rates of evaporation, moderate to minimal rainfall, strong summer winds, and low moisture content. It is acknowledged as one of Pakistan's warmest regions, with an extended summertime that lasts from May to October (Farooq et al., 2010).

Study Animals

The study included Sipli sheep (n = 60) raised at the university farm, all of which appeared clinically healthy. An intensive farming system is being implemented for the animal rearing. They are fed in stalls with a concentrate ration that contains about 15% crude protein and freshly chopped seasonal fodder. In addition, depending on demand, maize silage and wheat straw is also provided. The fresh and pure drinking water is always made available for them. Regular vaccination and deworming are conducted at the farm in addition to routine veterinary inspections. Routine hematological evaluations, combined with thorough physical examinations and input from farm staff, confirmed that all animals involved in the study were clinically healthy and exhibited no indications of illness.

The Sipli is a native sheep breed of Pakistan with somewhat long and thin tail, medium-sized body, with males weighing an average of 32.8 kg and females 29.2 kg. They produce 0.2-0.4 L of milk per day. Its head and ears are light brown or white, with its white body coat. Its ears extend to a length of approximately 15 centimeters, and its medium-sized head features a flat nose. It is primarily raised for the production of mutton and wool by the nomadic herders of Cholistan desert of Southern Punjab, Pakistan (Idris et al., 2024a; 2024b; Sharif et al., 2024; 2025).

Blood Sampling

Blood collection was performed by trained personnel, with each animal gently restrained during the procedure. A total of 5 mL of blood was aseptically drawn from the jugular vein using sterile technique. The samples were collected into EDTA-coated vacutainer tubes (TUBER® Vac EDTA K2, Australia) with purple caps, each containing 0.5 mL of 1% EDTA solution to prevent coagulation. Blood samples were brought to the laboratory in ice box and analyzed within 4 hours.

White Blood Cell Counting

The WBC counting was conducted by following techniques:

a) ***Manual method:*** White blood cell (WBC) counts were determined using a Neubauer hemocytometer (MARIENFELD, Germany), following the standard procedure described by

Berkson et al. (1935), with minor modifications. Turk's solution (SDL Scientific Enterprise, Pakistan) served as the diluting agent. Two dilution ratios, 1:20 and 1:40, were employed for the analysis and designated as WBC-1 and WBC-2, respectively.

b) *Automated method:* Automated WBC counts were conducted using two multispecies veterinary hematology analyzers - Rayto RT-7600Vet and Biobase BK-5000Vet (China) - referred to in this study as WBC-R and WBC-B, respectively. Both devices are 3-part differential analyzers that classify WBCs into lymphocytes, intermediate cells, and granulocytes. These analyzers support hematological evaluation across 13 predefined species (including sheep) and four user-defined species. Given that sheep is among the predefined options, this default species setting was utilized for the current analysis.

Key operational specifications of both analyzers include a minimum whole blood requirement of 9.8 µL, a pre-diluted volume of 20 µL, and a test completion time of approximately one minute. They function optimally within a temperature range of 15-35°C and relative humidity of 10-90%. Each analyzer generates 23 hematological parameters along with histograms for WBCs, red blood cells (RBCs), and platelets (PLTs). The systems employ specific reagents - lysing agents, diluents, and cleaning solutions - supplied by the manufacturers to ensure accurate results, as per their user manuals. Additionally, reference intervals can be manually adjusted for individual species, and any values outside the normal range are automatically flagged in the complete blood count (CBC) report (Dokumen, 2020).

Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics (Version 20) and GraphPad Prism (Version 8.0.1). Outlier detection was performed through visual inspection in conjunction with the Shapiro-Wilk test to evaluate normality. Data were stratified based on sex (females, $n = 43$; males, $n = 17$) and age categories (≤1 year, $n = 10$; 1–2 years, $n = 35$; >2 years, $n = 15$). Reference intervals (RIs), defined as the 25th to 90th percentiles, were established in accordance with the guidelines of the American Society for Veterinary Clinical Pathology (Friedrichs et al., 2012), utilizing the Reference Value Advisor software (version 2.1; available at: http://www.biostat.envt.fr/reference-value-advisor).

Comparisons between manual counts and automated hematology analyzers were assessed using one-way ANOVA followed by Duncan's multiple range post-hoc test, with significance determined at $P \leq 0.05$. Pearson's correlation coefficient was employed to examine the relationships between WBC counts derived from different methods, while linear regression was applied to develop predictive equations.

To evaluate the level of agreement between the two automated analyzers, three complementary methods were used: Bland-Altman analysis (B & A), Cronbach's alpha, and the Intraclass Correlation Coefficient (ICC). Furthermore, Lin's Concordance Correlation Coefficient (LCCC) was utilized to assess both accuracy and precision of the analyzers' measurements.

RESULTS AND DISCUSSIONS

Multispecies automated hematology analyzers are intricate instruments validated by manufacturers under ideal conditions before market release, following national or regional regulatory frameworks such as those of the FDA (USA) and CE Mark (EU). However, their routine analytical and diagnostic performance often shows significant variability. To ensure accurate and reliable results in daily use, international guidelines provided by organizations like American Society for Clinical Veterinary Pathology (ASVCP), the Clinical Laboratory Standards Institute (CLSI), and the International Organization for Standardization (ISO) outline verification criteria, including precision, accuracy, linearity, and comparability. These guidelines also emphasize the importance of performance evaluation, which is at the discretion of clinical laboratories and their personnel (Farooq et al., 2023a; 2023b). This study, is the first of its kind, which incorporated indigenous Sipli breed of sheep of Pakistan, for assessing the performance evaluation of 3-part multispecies hematology analyzer for WBC counting, in comparison to manual quantification methods (through hemocytometer) in a method-comparison analysis.

The results of normality testing in terms of Shapiro-wilk value, kurtosis, skewness and CV% are given in Figure 1.

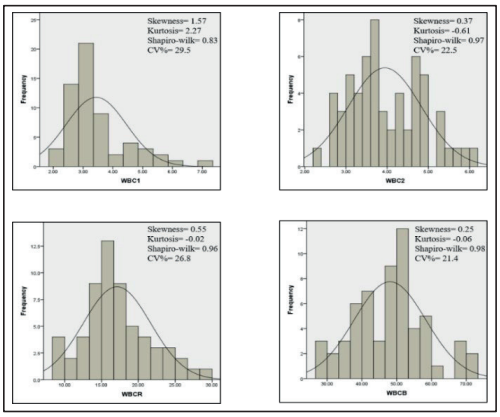


Figure 1. Histograms depicting the distribution of white blood cell (WBC) counts in Sipli sheep (n = 60), obtained through manual counting methods (WBC-1 and WBC-2) and two automated hematology analyzers (WBC-R and WBC-B). The plots illustrate the variability and spread of WBC values across all four methods, enabling visual comparison of data distribution patterns

In the present study, data of WBC count attained through both multispecies veterinary hematology analyzers (WBC-R and WBC-B) had lowest skewness and kurtosis which is in line with previous studies (Daves et al., 2024; Huang et al., 2023; Ma et al., 2023). Manual quantification of white blood cells using hemocytometry is inherently subjective and susceptible to variability due to operator skill level and inconsistencies in dilution techniques. Previous studies have demonstrated that automated hematology analyzers offer superior sensitivity, reliability, and predictive accuracy when compared to traditional manual methods such as hemocytometry (Clark et al., 2019; Lee et al., 2022; Oikonomidis et al., 2024).

The results for overall comparison of WBC count between manual methods (WBC-1 and WBC-2) and through multispecies automated hematology analyzers (WBC-R and WBC-B) are given in Table 1.

Results revealed that the mean (±SE) values for WBC-1 and WBC-2 were non-significantly different from each other whereas, WBC-R and WBC-B were significantly different (P≤0.05) from each other, and also differed significantly from WBC-1 and WBC-2.

Table 1. Descriptive statistics for white blood cell (WBC) counts obtained from apparently healthy Sipli sheep (n = 60) using manual methods (WBC-1 and WBC-2) and automated hematology analyzers (WBC-R and WBC-B). Parameters presented include overall mean ± standard error (SE), median, interquartile range (IQR), minimum and maximum values, reference interval (RI; 25th to 90th percentile), and 95% confidence interval (CI).

Methods	Mean ± SE	Median (IQR)	Range (Min.-Max.)	RI (25th-90th)	95% CI
WBC-1	3.44±0.13[a]	3.10(0.98)	4.90 (2.10-7.00)	2.80-5.19	3.17-3.70
WBC-2	3.94±0.11[a]	3.70(1.45)	3.70 (2.30-6.00)	3.22-5.20	3.71-4.17
WBC-R	17.09±0.59[b]	16.5(5.79)	19.92 (9.45-29.37)	13.84-24.60	15.91-18.28
WBC-B	48.07±1.32[c]	48.56(13.15)	44.95 (27.97-72.92)	40.28-60.58	45.41-50.73

[a, b, c] Superscripts indicate the significance at (P≤0.05) for different methods of WBC counting.

Considering the mean (±SE) values and RIs for WBCs of the present study, it was revealed that data of WBC-R was within the physiological range for sheep blood given elsewhere (Mostaghni et al., 2005; Rishniw & Pion, 2016). Similar results were attained for gender-based and age-based groups as well as shown in Tables 2 and 3, respectively. The WBC-R for overall and group-wise data was significantly (P≤0.05) higher (17.09±0.592×109/L) than WBC-1 and WBC-2, and lower than WBC-B, though within the normal physiological range. Similar results were attained for gender-based and age-based groups. The WBC count in another study (Abdel-Lattif & Al-Muhja, 2021; Zamfirescu et al., 2009) showed the mean (±SE) values of 18.60±0.69 and 15.41±1.21 which are nearly equal to the value obtained through WBC-R of present study. While, the lower values have also been reported for sheep WBCs being 9.13±0.59 and 7.3±1.8 (Ahmadi-hamedani et al., 2016; Frye et al., 2022). Such huge variation in WBC count for sheep inevitably depicts that starting from blood collection, transport and analysis till the use of various techniques (manual and/or automated) affect the count and need to be kept in strict consideration.

Table 2. Gender-Wise WBC Count as Deduced through Manual Methods (WBC-1 and WBC-2) and Hematology Analyzers (WBC-R and WBC-B) for Apparently Healthy Sipli Sheep (n = 60)

Methods	Mean ± SE	Median (IQR)	(Min.-Max.)	Range	RI (25th-90th)	95% CI
Males (n = 17)						
WBC-1	3.23±0.185[a]	3.00 (0.85)	(2.10-5.20)	3.10	2.80-4.64	2.84-3.62
WBC-2	3.76±0.252[a]	3.70 (1.70)	(2.30-6.00)	3.70	2.80-5.44	3.23-4.29
WBC-R	18.50±1.358[b]	17.18 (8.78)	(9.70-29.37)	19.67	14.82-27.25	15.62-21.38
WBC-B	48.83±3.229[c]	50.76 (19.21)	(27.97-72.92)	44.95	36.71-70.60	41.99-55.68
Females (n = 43)						
WBC-1	3.52±0.167[a]	3.20 (1.00)	(2.10-7.00)	4.90	2.80-5.42	3.18-3.86
WBC-2	4.01±0.126[a]	3.70 (1.20)	(2.60-5.80)	3.20	3.50-5.20	3.75-4.26
WBC-R	16.54±0.618[b]	16.33 (5.37)	(9.45-25.83)	16.38	13.50-22.10	15.29-17.79
WBC-B	47.77±1.372[c]	48.37 (12.44)	(28.67-67.48)	38.81	41.08-59.41	45.00-50.54

SE=Standard Error; IQR=Interquartile Range; RI=Reference Interval; CI=Confidence Interval; [a, b, c] Superscripts indicate the significance at (P≤0.05) for different methods of WBC counting within gender-based groups.

Table 3. Age-Wise WBC Count as Deduced through Manual Methods (WBC-1 and WBC-2) and Hematology Analyzers (WBC-R and WBC-B) for Apparently Healthy Sipli Sheep (n = 60)

Methods	Mean ± SE	Median (IQR)	(Min.-Max.)	Range	RI (25th 90th)	95%CI
G1 = up till one year						
WBC-1	3.23±0.278 [a]	3.00 (0.50)	(2.30-5.50)	3.20	2.82-5.32	2.60-3.86
WBC-2	3.89±0.230[a]	3.55 (1.30)	(2.90-4.80)	1.90	3.40-4.79	3.36-4.41
WBC-R	14.43±0.926[b]	15.02 (4.87)	(9.45-18.87)	9.42	11.89-18.72	12.33-16.52
WBC-B	48.81±2.837[c]	48.56 (6.26)	(32.41-67.48)	35.07	45.46-66.23	42.39-55.22
G2 = from one to two years						
WBC-1	3.45±0.191[a]	2.90 (1.00)	(2.10-7.00)	4.90	2.80-5.24	3.06-3.84
WBC-2	3.77±0.149[a]	3.60 (1.20)	(2.30-6.00)	3.70	3.10-5.02	3.46-4.07
WBC-R	17.98±0.821[b]	17.18 (5.90)	(9.46-29.37)	19.91	15.02-25.55	16.31-19.64
WBC-B	51.12±1.520[c]	51.40 (13.45)	(35.72-72.92)	37.20	43.31-63.25	48.03-54.21
G3 = above two years						
WBC-1	3.55±0.213[a]	3.40 (0.90)	(2.60-5.80)	3.20	3.00-5.02	3.09-4.01
WBC-2	4.38±0.232[a]	4.50 (1.50)	(2.70-5.80)	3.10	3.70-5.62	3.88-4.87
WBC-R	16.81±1.115[b]	15.40 (7.18)	(9.70-24.81)	15.11	13.56-23.26	14.42-19.20
WBC-B	40.47±2.745[c]	38.12 (12.70)	(27.97-70.03)	42.06	33.12-57.88	34.58-46.36

SE=Standard Error; IQR=Interquartile Range; RI=Reference Interval; CI=Confidence Interval; G1= up till 1 year; G2= from 1 to 2 years, G3= above 2 years.
[a, b, c] Superscripts indicate the significance at (P≤0.05) for different methods of WBC counting within age-based groups.

In contrast, the results obtained through manual methods (WBC-1 and WBC-2) in the present study showed the mean (± SE) values were lower than normal physiological range for sheep (3.44-3.94×109/L). Similarly, values attained through other hematology analyzer (WBC-B) showed the values were higher than normal physiological range for sheep (48.07×109/L). The lower values than normal physiological range obtained through manual methods could reasonably be clarified through the subjectivity of the hemocytometry method. Moreover, the higher values than normal physiological range obtained through hematology analyzer (WBC-B) may be because of the built-in capabilities of equipment (Clark et al., 2019; Hippel, 2007).

As the WBC count attained through multispecies automated hematology analyzer (WBC-R) was within normal physiological range for sheep in the present study, hence logilinear regression was implied between the data of WBC-R and the data obtained through other three methods i.e. WBC-1, WBC-2 and WBC-B (Figure 2).

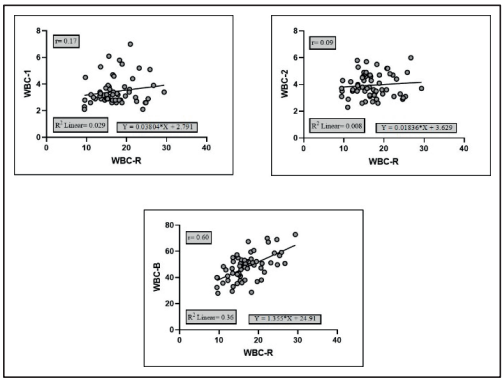


Figure 2. Scatterplots illustrating log-linear regression relationships between WBC counts obtained via the Rayto hematology analyzer (WBC-R) and three comparison methods in apparently healthy Sipli sheep (n = 60): a) manual method WBC-1, b) manual method WBC-2, and c) automated analyzer WBC-B. Each plot demonstrates the strength and pattern of association between WBC-R and the respective methods

A weak relationship was noticed between the WBC count attained through hematology analyzer (WBC-R) versus manual methods (WBC-1 and WBC-2) as indicated by weak r-values and r-square values. Moderately high level of relation was noticed only between WBC-R and WBC-B (r-value = 0.60; r-square value = 0.36; 36% probability). The results are in strong concordance to previous research works which have endorsed that manual quantification of cells (through hemocytometry) gives erroneous results and is prone to subjectivity whereas automated analyzers are precise and provide accuracy (Daves et al., 2024; Ma et al., 2023; Michael et al., 2022; Vis & Huisman, 2016). However, a weak correlation (36% probability) between the two automated multispecies hematology analyzers of the present study is not in line with previous studies which have reported a strong correlation between different hematology analyzers (Grebert et al., 2021; Vis & Huisman, 2016).

Automated multispecies hematology analyzers are widely regarded as the gold standard for assessing blood characteristics. In this study, the level of agreement between two such analyzers (WBC-R and WBC-B) was evaluated using B & A analysis, Cronbach's alpha, and ICC. The B & A plot (Figure 3) revealed a weak agreement between the analyzers, with a mean bias of -30.97 (upper limit: -14.56, lower limit: -46.77) and a standard deviation of bias of 8.37.

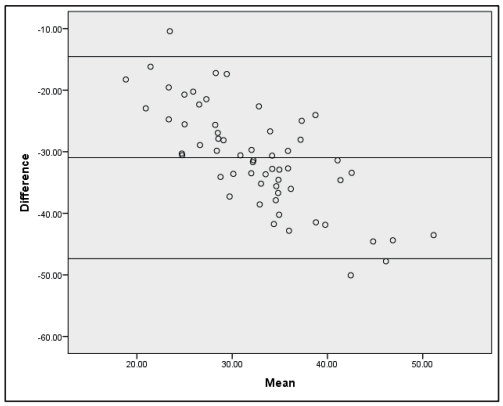


Figure 3. Bland-Altman scatterplot illustrating the agreement between WBC counts obtained from two automated hematology analyzers - WBC-R and WBC-B - in Sipli sheep (n = 60). The central horizontal line represents the mean difference (bias) between the two methods, calculated as –30.97. The upper and lower limits of agreement are depicted as –14.56 and –46.77, respectively, based on the standard deviation (SD) of the bias, which was 8.37.

Cronbach's alpha and ICC results (Table 4) further supported this finding, with the ICC value of 0.619 indicating moderate reliability

under a two-way random effects model for consistency. The LCCC reflected low accuracy (0.086), while precision was moderate (0.603). Additionally, the mean bias error (MBE) was calculated at 30.268, with WBC-B consistently reporting higher WBC counts compared to WBC-R. These findings contrast with prior studies that reported stronger agreement among different multispecies hematology analyzers, underscoring the variability in analyzer performance and the need for validation before clinical use (Brouwer et al., 2017; Cook et al., 2016; Michael et al., 2022; Rishniw & Pion, 2016).

Table 4. Cronbach's Alpha, Intraclass Correlation, Lin's Concordance Correlation Coefficient and Mean Bias Error (MBE) between WBC-R and WBC-B in Apparently Healthy Sipli Sheep (n = 60)

WBC-R vs WBC-B						
Intraclass correlation		95% CI	Cronbach's Alpha	Accuracy	Precision	MBE
Single measure	0.449	0.221-0.629	0.619	0.086	0.603	30.268
Average measure	0.619	0.363-0.773				

CI = Confidence Interval

CONCLUSIONS

The present study concludes that hemocytometer-based manual techniques for counting WBCs in sheep are likely inaccurate, as they tend to yield underestimated values.

Among the 3-part multispecies hematology analyzers evaluated in this study (Rayto RT-7600Vet and Biobase BK-5000Vet, China), the Rayto RT-7600Vet produced WBC counts closest to the physiological range for sheep. However, when using 3-part analyzers with predefined settings for sheep, caution is required, as their accuracy may vary.

To ensure reliable hematological assessment in sheep, it is essential to establish species-specific reference intervals (RIs) for each analyzer.

These findings provide valuable guidelines for research laboratories and clinical settings, particularly those with limited resources, emphasizing the cautious interpretation of results.

It is recommended that laboratories establish analyzer-specific reference intervals (RIs) and coefficient of variation (CV %) values for all hematological parameters.

Regular validation protocols should be implemented for both 3-part and 5-part hematology analyzers to ensure accuracy and reliability.

Additionally, RIs should be derived from larger populations of healthy animals to improve the precision and applicability of these techniques in clinical and research settings.

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ANIMAL WELFARE AND PROTECTION – UNDERSTANDING THE NATURE OF ANIMAL CRUELTY AND INTERPERSONAL VIOLENCE

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Abstract

Veterinarians as professionals play a major role in the wellbeing of their patients. Ensuring the animals` needs and healthcare, however, is not enough when the whole society should be involved in improving animal welfare and preventing maltreatment and animal cruelty. Our study focused on the veterinary practitioners as specialists who should be able to recognize types of animal abuse and understand its role in interpersonal violence. For this purpose, an anonymous written questionnaire was distributed among veterinarians in Bulgaria and Turkey. The results showed that the professionals` understanding on the nature of animal cruelty was influenced by respondents` demographics and over 80% of Bulgarian and Turkish veterinarians agreed on the ability of animals to experience emotions (P<0.05). The most recognized type of animal cruelty was physical abuse combined with neglect. The majority of the practitioners in both countries have awareness on the link between animal abuse and violence against people. These perceptions were affected to some extent by factors like gender and pet ownership (P<0.05).

Key words*: animal cruelty, animal welfare, interpersonal violence, veterinary practice.*

INTRODUCTION

Veterinarians are uniquely positioned to detect and intervene in cases of animal cruelty due to their interactions with both animals and their owners. The role of the veterinary profession extends beyond improving animal health to include public education and advocacy for animal welfare (Balkin et al., 2013). Animal cruelty and violence are not only ethical issues but also matters of public health and societal concern. Studies within the context of the relationship between animal cruelty and broader forms of violence, such as domestic violence, highlight the societal dimension of this issue (Ascione, 1999; Flynn, 2001).

Animal rights and animal welfare have become increasingly important topics in today's societal structure (Wu, 2022). The growing awareness of this issue necessitates that those various professionals, particularly veterinarians, play a critical role in combating violence against animals (Patterson-Kane et al., 2022). Veterinarians are not only responsible for safeguarding the physical health of animals but also serve as key actors in recognizing, reporting, and initiating legal proceedings in cases of animal abuse (Kara & Yılmaz, 2019). However, cases of animal abuse do not only affect animals; it is now a well-established fact that such situations are directly related to interpersonal violence, particularly against more vulnerable groups such as children and women (Davis et al., 2019). Numerous studies have shown that violence against animals is closely linked to domestic violence and other social issues (Kellert & Felthous, 1985; Patterson-Kane & Piper, 2009; Burchfield et al., 2022). Some authors (Baldry, 2003; DeGue & DiLillo, 2009; Cleary et al., 2021) argue that animal abuse is often associated with domestic violence and that animals are used as tools of control by perpetrators. In the recent years, the recognition of animal cruelty as an indicator and its connection to domestic violence has gained importance (Fitzpatrick et al., 2021), supported by the 'link' theory (Lockwood & Ascione, 1998). With regard to this, the detection of animal abuse cases is crucial for both victims of violence - animals and humans.

However, some research indicates that veterinarians may struggle to recognize animal

maltreatment and may be reluctant to intervene (Müller & Bonk, 2020). Their awareness varies across countries due to factors such as cultural norms and legal regulations (Merz-Perez et al., 2001). Another reason is that many veterinarians lack sufficient training or professional guidance on animal abuse (Tuna & Uygun, 2020). Additionally, most cases of animal abuse are concealed or misrepresented by the animals' owners, making it difficult for veterinarians to detect such situations (Harman & O'Neil, 2021).

In Bulgaria and Turkey, veterinary medicine is a respected field that contributes to animal health, public safety, and environmental sustainability. However, limited training on animal cruelty and differences in legal practices represent barriers to effective intervention. The study is aimed at assessing veterinary practitioners` current awareness on the problem, thus to emphasize the necessity to integrate animal protection education fully into veterinary curricula and continuous professional development programs.

MATERIALS AND METHODS

Design of the study

A cross-sectional study was conducted through a survey (an anonymous written questionnaire) administered to veterinary practitioners in Bulgaria and Turkey in online form with the support of the national veterinary associations. The survey was conducted in the period from January 2024 to August 2024 and all participants were informed about its voluntarily character and that by filling in the questionnaire they give their informed consent for participation. The survey included questions on the respondents` demographics, clinical practice characteristics, and their understanding of animal cruelty. A total of 445 veterinarians participated in the study, with 222 from Bulgaria and 223 from Turkey. The participants had different field of specialization, mainly working with pets, farm animals, and exotic species in urban and rural areas.

After collecting the results from the online questionnaire, all textual answers were converted into numerical values for further processing by the authors.

Statistical analysis

The data were analyzed using SPSS software (IBM SPSS-Inc., 2019, SPSS Reference Guide 26 SPSS, Chicago, USA). The methods used for analysis were descriptive statistics (frequency distribution) and chi-square test, the latter was applied to determine significant relationships between the respondents` variables and awareness levels. A threshold of $p < 0.05$ was considered significant. Visually, the results were presented in tables and diagrams (Excel, Windows 10).

RESULTS AND DISCUSSIONS

The respondents who participated in the study come from diverse background. All of them were qualified veterinarians from both genders and while the Bulgarian veterinary practitioners had almost balanced female: male ratio (BG 46.4% females and 53.6% males) which corresponded with the global trend of feminization of the profession (Irvine & Vermilya, 2010; Gordon et al., 2023), nearly two-thirds of the Turkish veterinarians were male (TR 35.6% females and 64.4% males), similar to the findings of Balaban & Güneş (2021) on the practitioners in Turkey (Table 1). Gender disparities in Turkey may reflect societal norms that could affect professional attitude to some extent. The majority of the respondents fall in the 30 to 60 age group (BG 64.4%; TR 73.9%) who appear to be veterinarians with more professional experience (FVE, 2023; Todorov & Balieva, 2024), while approximately one-third were between 25 and 29 years old (BG 31.1%; TR 24.9%) who could be described as newly graduated and very small proportions of the participants counted for other age categories.

Another indicator of importance to the veterinarians` demographics appeared to be the location of their clinic/dispensary. Most of the respondents practiced in clinics established in highly populated urban regions, as capitals or big cities and administrative centers (BG 86.9% in total; TR 97.2% in total), as described also by Gonschor et al. (2023), while very few of them were located in less populated small towns (BG 1.8%; TR 0.4%) or villages in rural areas (BG 10.8%; TR 2.4%).

Regarding the diverse range of animal species that the veterinarians were specialized in treating, the results showed that half of the respondents were small animals practitioners working with pet animals (BG 50.0%; TR 47.8%), in line with the conclusions by the FVE (2023) about the contribution of the companion veterinarians to the profession in Europe. Some of the participants of the survey worked predominantly with farm animals (BG 16.2%; TR 19.4%) and equal share of both nationalities had a mixed practice for small and large animals (BG 27.9%; TR 27.3%). As new branches of veterinary practice had become attractive in the recent decade (De Briyne & Iatridou, 2016) there were practitioners who worked with exotic animals (BG 3.2%) and wildlife (BG 2.7%) and others occupied with different responsibilities as for example working with stray dogs or race horses (TR 4.0%) (Table 1).

Table 1. Veterinary practitioners` demographics compared between Bulgarian and Turkish respondents

	Nationality			
Respondents`	Bulgarian		Turkish	
Demographics	Count	Percentage	Count	Percentage
Gender				
Female	103	46.4	90	35.6
Male	119	53.6	163	64.4
Age (years)				
24	6	2.7	N/A	N/A
25-29	69	31.1	63	24.9
30-60	143	64.4	187	73.9
61-64	2	0.9	N/A	N/A
65 and over	N/A	N/A	3	1.2
Practice location				
Capital city	22	9.9	121	47.8
City - Regional administrative center	129	58.1	62	24.5
City - Municipal administrative center	42	18.9	63	24.9
Town	4	1.8	1	0.4
Village	24	10.8	6	2.4
Type of practice				
Companion animal practice	111	50.0	119	47.0
Farm animal practice	36	16.2	49	19.4
Mixed practice	62	27.9	69	27.3
Exotic animals' practice	7	3.2	N/A	N/A
Wild animals	6	2.7	N/A	N/A
Other	N/A	N/A	10	4.0

*Values may not total 100% for each category because of non-responders and rounding of values.

As the veterinarians appeared to hold the sole professional responsibility to treat and advocate for the well-being of the animals, our respondents were fully in consent with the concept that animals as living beings could form and experience emotions (BG 82.0% "strongly agree", 13.1% "agree"; TR 85.0% "strongly agree", 13.8% "agree") (Figure 1). Their perceptions were fully in line with the implemented legislative framework on animal protection, covering the concept of the "five freedoms" (Özgür, 2007; Mellor, 2019; Robertson & Goldsworthy, 2022), including the humane treatment and veterinary care. Recent research confirmed that non-human animals should be perceived as sentient beings, having the ability to feel pain and distress (Coleman, 2008; Cornish et al., 2018). Some authors even suggested that demographic factors as gender for example could influence the perceptions on animal sentience and their emotions (Phillips et al., 2012; Tamioso et al., 2018). Such dependence was confirmed for both Bulgarian and Turkish veterinarians as a significant difference was found between gender and the respondents` perceptions of the ability of animals to experience emotions (All $\chi 2 = 12.567$; df = 4; p = 0.014). In fact, greater concern to animals was shown usually by women (Herzog, 2007).

High shares of both nationalities also stated that as professionals they were fully aware of the nature of animal cruelty (BG 79.3% "strongly agree", 19.8% "agree"; TR 58.9% "strongly agree", 28.9% "agree"). This finding held great importance as the veterinarians should at early stage recognize various acts of animal abuse in order to prevent further violence in society (Morris, 2010; Lachance, 2016).

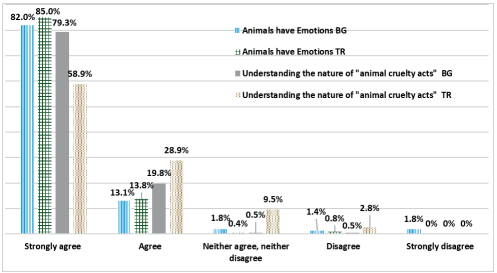


Figure 1. Veterinarians` understanding on the nature of animal cruelty and their agreement with the ability of animals to form emotions

Although the respondents declared high comprehension on animal cruelty in general, it

appeared that their knowledge on the main types of animal abuse differed a lot (Figure 2). All acts of animal cruelty were defined under four main categories (Rowan, 2006; Munro & Munro, 2008) but the respondents recognized predominantly physical abuse (BG 36.5%; TR 2.0%), psychological abuse (BG 8.6%; TR 0.4%) and neglect (BG 6.8%; TR 1.6%), while sexual abuse remained almost non-existent (BG 2.7%; TR 0%). At the same time almost all of the Turkish practitioners (TR 96.0%) and nearly 40% of their Bulgarian colleagues (BG 39.6%) confirmed their high awareness on the combination of maltreatment of animals encompassing both physical abuse and neglect. This fact could be explained with the more visible signs of violence against the animals like battered pets (Newberry, 2018) or starved and hoarded animals (Williams, 2014).

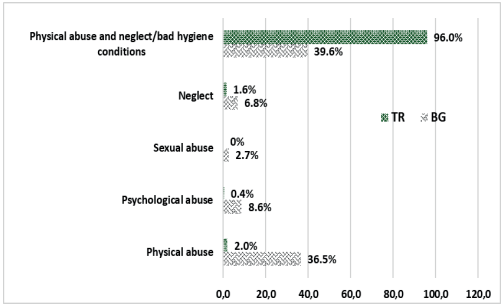


Figure 2. Awareness of veterinary practitioners in Bulgaria and Turkey of the types of animal abuse

Once more, gender was found to be a significant factor increasing the respondents` awareness of the four different types of cruelty towards animals (All $\chi 2 = 20.732$; df = 3; p = 0.000), with female veterinarians showing more ethical concerns with regard to animal abuse (Williams et al., 2022). It appeared that the location of the veterinary practice could influence significantly the practitioners` awareness of different type of animal abuse (All $\chi 2 = 111.848$; df = 20; p = 0.000) as the urban settlements were likely to increase the rate of animal cruelty offences due to social and neighbourhood disorganization (Reese et al., 2020). On the other hand, some rural areas were reported to have high rate of neglect and hoarding cases (Whitfort et al., 2021).

In some countries veterinarians work closely with other professionals in order to help the victims suffering from abuse (Paterson et al., 2024). Particular guidelines were developed and introduced to the veterinary specialists to help them in recognizing the signs of violence towards animal and human victims (AWF, 2016). However, as emphasized by Niemiec & Kogan (2024), the insufficient level of knowledge and understanding of interpersonal violence could appear as a serious barrier to addressing the violations of human and animal welfare. The results from the survey in the two neighbouring countries - Bulgaria and Turkey, the majority of the respondents stated very high level of awareness regarding the link between animal abuse and domestic violence (BG 58.1% “strongly agree”, 27.0% “agree”; TR 70.0% “strongly agree”, 22.9% “agree”) (Figure 3). These findings corresponded with other studies as Oellig et al. (2024) reported about 90% awareness of this interpersonal violence among licensed veterinarians. Regarding the high percentage of Turkish practitioners, it could be attributed to recent efforts in Turkey to protect stray animals, which had become a significant national agenda item.

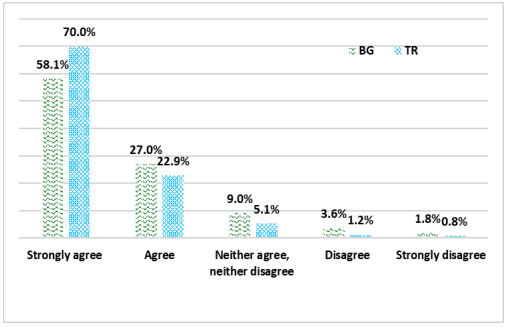


Figure 3. Recognition of the connection between acts of cruelty against animals and acts of violence against people (children, women, elder people)

Research showed that there were some demographic characteristics that could affect the comprehension of the interconnected human and animal abuse (Joo et al., 2020) which was confirmed for the Bulgarian and Turkish veterinarians. The results indicated that gender influenced the recognition of the link between animal abuse and interpersonal violence (All $\chi 2 = 14.071$; df = 4; p = 0.007) with women being more perceptive.

CONCLUSIONS

The survey about the Bulgarian and Turkish veterinary practitioners showed varying levels of awareness on the specific types of animal abuse and interpersonal violence influenced by different factors. The findings revealed that while both groups of respondents presented very high levels of comprehension on animal sentience and emotions, with good understanding of the nature of animal cruelty, their awareness of specific types of abuse and their role in identifying domestic violence varies. The results indicated that gender and practice location influenced veterinarians' perceptions to animal cruelty. Female veterinarians showed greater ethical concern, and those practicing in urban areas were more aware of cases of animal abuse.

However, their perceptions of the particular acts of violence against animals and its role as an indicator for domestic violence, made clear the necessity of implementation of advanced training modules and continuing education programs for veterinarians on animal protection and recognition of animal abuse. Additionally, interdisciplinary cooperation and joint efforts with other professionals like law enforcement authorities and social workers should be considered in promoting animal welfare and societal well-being under adequate intervention strategies.

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THERMAL RESPONSE TO COLD STRESS IN TWO DIVERGENT STRAINS OF HOLSTEIN DAIRY CALVES – PRELIMINARY RESULTS

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Abstract

Cattle carrying the SLICK mutation (c.1382del; rs517047387) were shown to exhibit increased resistance to heat stress, with limited research on thermal response of carriers under cold stress. The objective of this study was to evaluate the response of un-weaned calves to cold stress exposure in two divergently selected Holstein strains, namely descendants of SLICK carrier bulls (experimental group - EG, n = 9) compared to those of Romanian Black and White bulls (controls - CG, n = 11). Calves were monitored during a cold weather episode of 3 consecutive days, with lower critical temperatures (<5°C for >12h/day), for infrared thermography (IRT), growth rates and cortisol levels. Average daily gains were of 792±110 g in the EG and of 928±150 g in CG group (P>0.05). IRT data showed significant differences between groups during the first day of the cold weather event, for both orbital (26.48±0.444°C in EG vs. 28.74±0.472°C in CG calves, P≤0.01) and nasal (18.23±0.820°C in EG vs. 21.05±1.200°C G in controls, P≤0.10) regions. Results suggest that calves sired by SLICK carrier bulls are exhibiting lower growth rates and have lower IRT-body temperatures in response to cold stress events.

Key words*: cold stress, dairy calves, growth rates, infrared thermography, SLICK gene.*

INTRODUCTION

Europe is the fastest warming continent in the world, and as a result climate risks are threatening food security, ecosystems, water resources, farmers' financial stability and animals' productivity (EEA, 2024). The mean annual temperature in Europe was 2.1°C warmer in the last 10 years, compared to the pre-industrial period. As a result, climate change adaptation measures are critical to increase resilience and reduce risks in agriculture, including the dairy cattle sector.

Heat stress was shown to negatively affect cattle production, reproduction, health and general performance, leading to substantial economic losses (West, 2003; Wang et al., 2018). Moreover, heat tolerance in dairy cattle was found to have a negative genetic correlation with milk production parameters (Aguilar et al., 2009; Pryce et al., 2022).

In cattle, the prolactin receptor gene (PRLR), has undergone several mutations, collectively referred to as SLICK mutations. Cattle carrying at least one allele of the *SLICK* mutation have phenotypes characterized by a short, sleek hair coat (Dikmen et al., 2014).

SLICK mutations are prevalent in cattle breeds originating from the Caribbean basin and South America (Porto-Neto et al., 2018; Florez Murillo et al., 2021). The first SLICK mutation described was SLICK1 (c.1382del; rs517047387), found in the Senepol breed originating on the Caribbean Island of St. Croix (Littlejohn et al., 2014), which was recently introduced throughout introgression in the Holstein breed (Sosa et al., 2022).

Dairy cattle that are heterozygous for the SLICK1 mutation have demonstrated superior capabilities to regulate body temperature during intense heat stress exposure episodes (Eisemann et al., 2020; Landaeta-Hernandez et al., 2021).

The main significance of the SLICK1 mutation for the dairy industry is that carrier cows produce significantly more milk during intense heat stress, compared to cows without the mutation (Dikmen et al., 2014). While the

SLICK1 mutation was shown to improve thermotolerance to heat stress in carriers, consequences on the physiological responses under cold stress exposure are not yet documented.
The objective of this study was to evaluate the response of un-weaned calves to cold stress exposure in two divergently selected Holstein strains, namely descendants of SLICK1 carrier bulls, compared to those of Romanian Black and White bulls.

MATERIALS AND METHODS

The study was carried out at the Experimental Farm of the Research and Development Institute for Bovine Balotesti - Romania, between December 2024 and January 2025. Where two experimental groups were set-up, as follows: Group I - calves descendants of two SLICK carrier bulls crossed with Romanian Black and White cows (experimental group - EG, n = 9); Group II - purebred Romanian Black and White calves (controls - CG, n = 11). Both calves groups were managed under identical rearing conditions, and were monitored during a cold weather episode of 3 consecutive days, with temperatures of < 5°C, for +12 h/day (limits were corrected for air speed, and ranged between -3.9°C and +1.2°C), relative humidity 73-82% and wind speeds between 0.2 and 4.9 m/s. Temperature, humidity and air speed were recorded continuously using a Wi-Fi Sencor® (1xCR2032 model) weather-station, located in close proximity to the calves hutches. As reference values for setting-up the lower critical temperature values (LCT) for un-weaned calves, thresholds designed by AHDB UK (2024) were used.
Calves from the two experimental groups were balanced for age and body weight at the start of the experiment (EG 69.78±5.14 kg, CG 70.90±6.60 kg, *P = 0.8378*).
At birth all calves received 4 kg of colostrum per day, during their first 3 days of life and were kept in a maternity barn compartment. At 4-5 days of life, all calves were moved and housed in outside individual hutches (2.0 x 1.2 x 1.4 m), using deep straw bedding (1.5-2 kg/calf).
Suckling calves received a diet consisting out of 6 kg of milk, offered into two equal meals/day (at 12 hours intervals, 6:00 & 18:00). After the age of 10 days, calves had *ad libidum* access to fresh water, starter concentrates (18% CP) and alfalfa hay, until the age of 90±7 days, when weaning took place.
Infrared thermography (IRT) readings were taken using a FLIR ONE Pro LT mobile camera (19,200-pixel, range −20° to +400°C) and FLIR Systems INC© image processing software. Temperature measuring points were the lacrimal caruncle of the eye in the orbital region (*regio orbitalis*) and at the nasal region (regio nasalis), the IRT pictures being taken from a distance of 0.8–1.2 m in front of the calves (Figure 1), the three measuring times were at 6:00 AM (method described in detail by Mincu et al., 2023), right before the morning milk feeding.
Calves were weighted using a MP 800 - EziWeigh 7i® scale, at the beginning of the cold episode, at 7 and 28 days.
Blood samples were collected on the 3rd day of the cold exposure episode, and cortisol levels were analysed using the immune-enzymatic assay ELISA method (Stat Fax 2200-2600®).

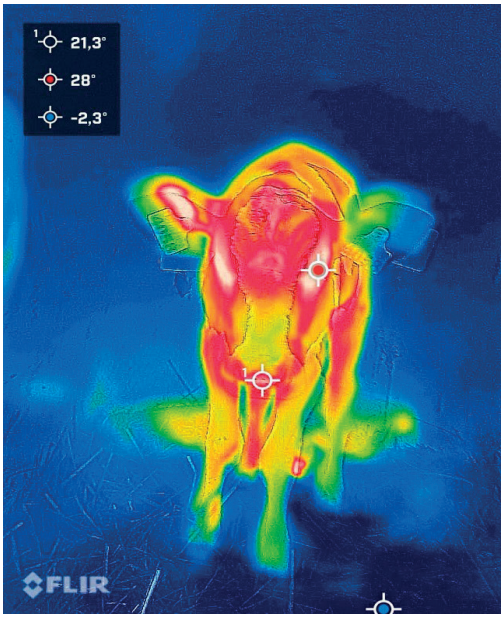


Figure 1. IRT reading for *regio orbitalis* and *regio nasalis* in a dairy calf during experiment (original photo)

Statistical analysis included descriptive statistics and the Mann-Whitney U test to determine differences between the two groups, using MiniTab17® software. Differences were considered significant at a level of P≤0.05, while the tendency for significance was set at P≤0.10.

The current experimental design was in accordance with the EU Directive 2010/63/EU on Experimental Animal Protection.

RESULTS AND DISCUSSIONS

Average daily gains of calves during the experiment were of 792±110 g/day in the EG and of 928±150 g/day in CG group (Table 1). Although the Romanian Black and White purebred calves distinctively outperformed the SLICK1 sired calves, differences were not supported statistically (P>0.05). The lack of statistical significance, could be attributed to the relative low number of subjects included in the study. However, differences in average daily gains of over 130 g/day are of importance, showing an impaired growth in SLICK1 sired group, given that the two groups had similar body live weights at the start of the experiment, and that they all received the same milk feeding diet.

Results on growth rates are in agreement with those previously published by Coffey et al. (2006), on differences in growth rates between divergently selected Holstein strains.

Hearth girth between the two groups was not significantly different (P>0.05) at the end of the experimental trial.

IRT data showed significant differences between the two groups during the 1st day of the cold weather event, for both orbital (26.4±0.44°C in EG *vs.* 28.7±0.47°C in CG calves, P≤0.01) and nasal (18.2±0.82°C in EG *vs.* 21.0±1.20°C G in controls, P≤0.10) regions (Table 2).

Furthermore, a tendency towards significance (P = 0.0873) was observed on 2nd day of the cold exposure event, for the nasal region. Generally, SLICK1 sired calves, had lower IRT readings for all three days of the experiment, in both anatomical regions.

Differences observed between the two groups during the 1st day of the naturally occurring cold event could be attributed to a delay in the activation of thermoregulation mechanisms in SLICK1 sired calves, in order to compensate for hypothermia.

Moreover, considering the innate ability of young ruminants to prevent a drop in the core body temperature, especially when severe hypothermia persists for extended periods of time, overcompensation triggering mechanisms could have been activated, leading to vasoconstriction as a reaction to prevents heat losses, and thus the SLICK1 sired calves had lower IRT readings, compared to the Romanian Black and White purebreds. This second hypothesis is supported by previous authors describing extensively the neonatal calves coping mechanisms to cold weather (Bertoni et al., 2020; Mota-Rojas et al., 2021).

Furthermore, our results regarding differences between the two anatomical measuring regions are consistent with those previously reported by Mota-Rojas et al. (2022), which found that IRT responses measured in various thermal windows differ, while the IRT temperature of the lacrimal gland shown the least temperature variation in bovines.

Blood cortisol levels were higher in SLICK1 sired calves, on average of 98.3±18.7 ng/ml, compared to Romanian Black and White purebred calves, which registered on average 67.6±14.9 ng/ml on the 3rd day of the cold weather event (Table 3). Although the blood cortisol levels in SLICK1 sired calves was on average higher 1.5 folded, compared to the local Holstein strain calves, there was no significant difference (P>0.05) between the two genotypes.

While cortisol levels in cattle are most commonly used as an indicator for welfare (Tamminen et al., 2021), being regarded as one of the main stress biomarkers, recent research by Kim et al. (2023) showed an elevation of cortisol levels in both beef calves and fattening steers, during cold stress exposure.

We are aware that the study has two limitations. The first is the less than desired number of subjects/calves included in the experimental groups, given that the SLICK1 mutation was just recently introduced in Romania. The second is represented by the short duration of the cold challenge, which might not elicit the full range of physiological responses in calves. To overcome these

shortcomings, for our future research trials on this subject, we plan to include a higher number of calves, as well as to monitor their health and growth performances for longer periods of time.

Our research trial suggests that when divergently selected cattle strains are introduced to new farming environments, significant attention should be given to un-weaned calves, in order to promote growth rates, general adaptation, health and animal welfare, taking into account practices such as the use of calf-jackets, increasing the daily milk intake, proper bedding and housing design.

Table 1. Averages and dispersion indices for average daily gain (g/day) and hearth girth (cm) in SLICK and non-SLICK un-weaned dairy calves during the cold stress period

Item	Genotype	Mean ± SEM	St. Dev.	Coef. Var.	Min.	Max.
Average daily gain (ADG, g/day)	SLICK	792±110	331	41.78	375	1250
	Non-slick	928±150	475	51.20	125	1750
Heart girth (cm)	SLICK	101.89±2.390	7.18	7.05	94.00	113.00
	Non-slick	102.20±3.420	10.81	10.58	85.00	94.00
Statistical differences						
ADG in SLICK vs. non-slick calves			P = 0.4840, *NS*			
Heart girth in SLICK vs. non-slick calves			P = 0.8697, *NS*			

Note: P>0.05 NS

Table 2. Averages and dispersion indices for orbital and nasal IRT data readings during the 3 days of cold weather exposure (°C)

Day	IRT Region	Genotype	Mean ± SEM	St. Dev.	Coef. Var.	Min.	Max.
I	Orbital	SLICK	26.48±0.444	1.332	5.03	24.70	28.40
		Non-slick	28.74±0.472	1.564	5.44	25.70	25.70
	Nasal	SLICK	18.23±0.820	2.460	13.49	14.10	21.40
		Non-slick	21.05±1.200	3.960	18.83	13.40	26.50
II	Orbital	SLICK	27.90±0.876	2.627	9.42	25.10	34.50
		Non-slick	29.245±0.920	3.051	10.43	23.20	33.30
	Nasal	SLICK	17.98±1.620	4.870	27.10	7.30	24.90
		Non-slick	21.51±1.240	4.100	19.05	12.70	26.90
III	Orbital	SLICK	33.28±0.858	2.573	7.73	28.90	36.10
		Non-slick	31.55±1.210	4.000	12.67	21.90	36.10
	Nasal	SLICK	24.02±1.970	5.900	24.55	16.50	33.50
		Non-slick	26.81±0.892	2.959	11.04	21.20	30.70
Statistical differences							
Day I, orbital region SLICK vs. Non-slick			**P = 0.0049, ****				
Day I, nasal region SLICK vs. Non-slick			P = 0.0681, *T*				
Day II, orbital region SLICK vs. Non-slick			P = 0.1596, *NS*				
Day II, nasal region SLICK vs. Non-slick			P = 0.0873, *T*				
Day III, orbital region SLICK vs. Non-slick			P = 0.3612, *NS*				
Day III, nasal region SLICK vs. Non-slick			P = 0.2871, *NS*				

*Note: P>0.05 NS; P≤0.01 **; Tendency towards significance (T) P≤0.10*

Table 3. Averages and dispersion indices for blood cortisol levels during the 3rd days of cold weather exposure (ng/ml)

Item	Genotype	Mean ± SEM	St. Dev.	Coef. Var.	Min.	Max.
Blood cortisol level (ng/ml)	SLICK calves	98.3±18.7	56.2	57.15	30.7	182.4
	Non-slick calves	67.6±14.9	47.2	69.78	26.1	144.5
Statistical differences						
Differences in blood cortisol levels in SLICK vs. Non-slick calves			0.3689, NS			

Note: P>0.05 NS

CONCLUSIONS

The evidence from this study suggests that calves sired by SLICK1 carrier bulls are exhibiting lower growth rates, have lower IRT-body temperatures, and show more elevated levels of blood cortisol in response to cold stress events, compared to purebred calves from the local Romanian Black and White Holstein strain.

This study is the first step towards enhancing our understanding on SLICK1 sired calves adaptation to temperate climatic conditions, with a focus on physiological responses to cold stress events during the first 3 months of age.

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SURGE IN FOODBORNE OUTBREAKS AND FATALITIES IN THE EU, A 2008-2022 OVERVIEW OF ZOONOTIC DISEASES, EMERGING THREATS AND WAYS OF MITIGATION

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Abstract

This article examines the significant surge in foodborne outbreaks (FBOs) and fatalities within the European Union from 2008 to 2022, focusing on zoonotic diseases and emerging threats. We highlight the increasing incidence of FBOs linked to pathogens such as Salmonella, Listeria, and Campylobacter, exacerbated by changing agricultural practices, global trade, and climate change. The review identifies critical challenges in food safety management, including gaps in surveillance systems and the need for improved risk assessment methodologies. In addition, this article considers a range of effective mitigation strategies, including traceability, public health education, and regulatory compliance. The findings underscore the necessity for a coordinated collective action to reduce the impact of foodborne zoonoses on public health in the EU.

Key words*: Campylobacter, food safety, Listeria, Salmonella, zoonotic diseases.*

INTRODUCTION

Zoonoses pose significant public health challenges globally. Surveillance efforts are essential to identify trends, mitigate risks, and implement effective control measures. This literature review examines the findings from the 2008 to 2022 reports published by the European authorities, which provide a comprehensive overview of zoonotic disease surveillance across Europe.

The European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) data reveal a consistent pattern in zoonotic disease reporting over the 15-year period from 2008 to 2022, with *Campylobacter, Salmonella, Listeria* and *E. coli* remaining the most frequently reported pathogens responsible for food-borne illnesses.

In 2010, *Campylobacter* spp. was estimated to have affected the health of 96 million people. Worldwide, 4.5 billion cases of infectious diarrhea and 1.6 million deaths are reported annually due to the consumption of contaminated food and water (Pires et al., 2015).

The European Union has faced a concerning escalation in foodborne outbreaks and associated fatalities over the past decade, with 2022 marking a pivotal year. Reported foodborne outbreaks surged by 43.9% compared to 2021, accompanied by heightened hospitalizations and the highest fatality count in a decade.

Listeria monocytogenes and *Salmonella* emerged as primary drivers of severe outcomes, while Campylobacteriosis and salmonellosis remained the most prevalent zoonotic infections. Concurrently, climate-sensitive pathogens like West Nile virus exhibited explosive growth, with cases increasing over 600% in 2022, underscoring the interplay between environmental shifts and epidemiological risks. The 15 years trends analysis, identifies emerging threats, and propose mitigation strategies, with a focus on the transformative potential of geospatial data in reshaping epidemiological surveillance.

MATERIALS AND METHODS

To gain a complete understanding of the surge in foodborne outbreaks and fatalities in the EU,

a meticulous analysis of zoonotic diseases, emerging threats, and potential mitigation approaches was made. Data collection was based on scientific reports from the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), as well as several peer-reviewed scientific articles.

The search was guided by specific keywords and phrases such as: *zoonotic diseases in the EU*, *foodborne outbreak trends (2008-2022)*, *emerging pathogens in food supply chains*, and *food safety mitigation strategies*. This approach was designed to capture the multifaceted aspects of the topic.

The reports were considered and reviewed based on the following: publication date (published between 2008 and 2022 to align with the review's scope), relevance (focus on zoonotic diseases, foodborne pathogens, and associated risk mitigation strategies within the EU), language (articles available in English to maintain consistency in analysis), and scientific rigor: governmental or EU agency reports, and reputable scientific articles.

Studies that did not directly address foodborne outbreaks or zoonotic diseases within the specified timeframe or region were excluded from the review.

Data extraction and analysis were conducted through a structured process. Reports were grouped based on themes such as major zoonotic pathogens (e.g., *Salmonella, Campylobacter*), emerging threats (e.g., antimicrobial resistance, climate-related effects), and mitigation measures (e.g., farm-to-fork approaches, policy interventions).

A thematic analysis was conducted, and trends, patterns, and gaps in the literature were identified. For instance, the role of climatic changes in influencing pathogen prevalence or the effectiveness of policy reforms in controlling outbreaks.

Each study was summarized, focusing on its contributions to understanding the causes, trends, and mitigation strategies related to foodborne outbreaks. Insights were synthesized, highlighting both consistent findings (e.g., the dominant role of *Salmonella* and *Campylobacter* in outbreaks) and contrasting perspectives (e.g., debates on the cost-effectiveness of specific mitigation strategies).

The review uncovered critical findings, including the persistent dominance of *Salmonella, Listeria monocytogenes*, and *Campylobacter* in EU foodborne outbreaks; the growing impact of climate change, globalization of food supply chains, and antimicrobial resistance on food safety; the importance of integrated approaches, such as the "One Health" framework, which links human, animal, and environmental health to combat zoonotic risks effectively.

RESULTS AND DISCUSSIONS

The surveillance data from 2008 to 2022 shows important trends in the three main zoonotic agents monitored by EFSA and ECDC (Figure 1).

Campylobacteriosis has consistently remained the most frequently reported zoonotic infection across the EU throughout this period, with cases initially rising from 190,566 in 2008 before gradually declining in recent years. In 2008, *Salmonella* was already noted as the second most reported zoonotic infection, with 131,468 cases, representing a 13.5% decrease from the previous year and continuing a downward trend that had begun earlier.

Table 1. Number of confirmed cases reported for the three principal zoonotic pathogens (original)

Year	*Campylobacter* spp.	*Salmonella* spp.	*Listeria monocytogenes*
2008	190,000	150,000	1,500
2009	195,000	145,000	1,600
2010	200,000	140,000	1,700
2011	210,000	135,000	1,800
2012	220,000	130,000	1,900
2013	230,000	125,000	2,000
2014	240,000	120,000	2,100
2015	235,000	117,000	2,000
2016	230,000	115,000	2,050
2017	225,000	113,000	2,100
2018	220,000	110,000	2,150
2019	215,000	108,000	2,200
2020	210,000	106,000	2,250
2021	205,000	104,000	2,300
2022	200,000	102,000	2,350

This table illustrates the overall trends. *Campylobacter* cases initially increased from 2008 to 2014, reaching a peak of 240,000 cases before beginning a consistent decline. *Salmonella* has shown a steady downward

trend throughout the entire period, decreasing from 150,000 to 102,000 cases. In contrast, *Listeria*, while occurring at much lower rates, has shown a gradual but consistent increase from 1,500 cases in 2008 to 2,350 in 2022.

Analysis of recent trends (2013-2022)

The comparative graph in Figure 1 also illustrates the trends for the three main zoonotic pathogens over the most recent decade (2013-2022). This clearly demonstrates the substantial difference in case numbers between the pathogens and their distinct trajectories.

Campylobacter spp. shows a declining trend after peaking around 2014. From approximately 240,000 cases in 2014, numbers gradually decreased to about 200,000 by 2022, representing approximately a 17% reduction over this period. Despite this decline, campylobacteriosis remains firmly established as the most frequently reported zoonotic infection in the EU, with case numbers nearly double those of *Salmonella*.

Salmonella spp. showed a consistent downward trajectory throughout the decade. Starting from approximately 125,000 cases in 2013, numbers declined to around 102,000 by 2022, representing an 18% decrease. This continued reduction builds upon earlier successes, as documented in the 2008 report which had already noted a significant 13.5% decrease from the previous year.

Listeria monocytogenes presents a different pattern altogether. With significantly lower case numbers, *Listeria* nonetheless shows a concerning upward trend, increasing from approximately 2,000 cases in 2013 to 2,350 by 2022.

This represents a 17.5% increase, making it the only one of the three main zoonotic agents to show an increasing trend during this period.

Public health implications, control measures and factors influencing disease trends

The declining trends in *Campylobacter* and *Salmonella* infections likely reflect successful implementation of control measures across the EU. For *Salmonella* specifically, the 2008 report highlighted the implementation of a new EU Commission program to reduce prevalence in laying hens, noting that 20 Member States (MSs) had already met their reduction targets for that year.

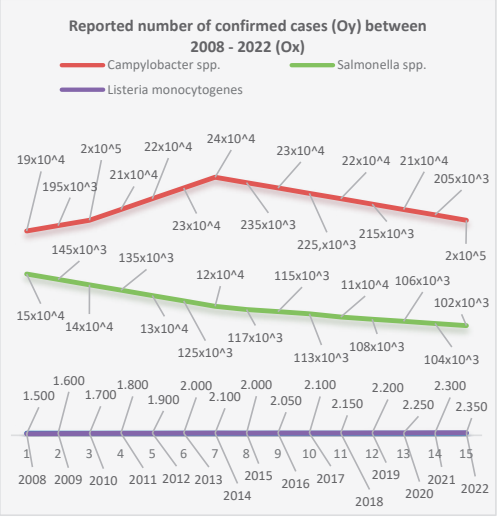


Figure 1. The three main zoonotic agents monitored by EFSA and ECDC and reported numbers (original)

The report suggested this could be the reason for decreasing *Salmonella enteritidis* infections in humans, as eggs are a known primary source for these infections.

While both major pathogens show decreasing trends, the 2022 report indicates that between 2021 and 2022, notification rates for campylobacteriosis and salmonellosis remained stable, suggesting the rate of improvement may be slowing. Simultaneously, there was a 15.9% increase in listeriosis cases between 2021 and 2022, continuing the concerning upward trend observed throughout the decade.

The EU's targeted approach to *Salmonella* control appears to have been particularly successful. The 2022 report notes that nineteen MSs and the United Kingdom (Northern Ireland) achieved all established targets in poultry populations for reducing *Salmonella* prevalence for the relevant serovars. This systematic approach to controlling *Salmonella* at the source has likely contributed significantly to the steady decline in human cases.

For *Campylobacter*, which is most frequently found in raw poultry meat, control measures have been more challenging to implement effectively, though the gradual decline in cases suggests some success in reducing contamination levels or improving consumer practices.

In 2014 and 2015, campylobacteriosis was a persistent challenge, being the most frequent zoonosis in the EU, with a constant rise since 2008. This trend reflects a high *Campylobacter* prevalence in the food chain, particularly in broiler meat, underscoring the critical need for enhanced food safety measures. The persistence of campylobacteriosis as a leading cause of foodborne illness indicates systemic challenges in controlling this pathogen within poultry production and processing systems.

Decimal logarithm values of zoonotic agent cases (2008-2022)

The Table 2 presents the decimal logarithm (log10) values of the reported cases for the three main zoonotic pathogens monitored by EFSA and ECDC from 2008 to 2022.

Table 2. Logarithmic zoonosis trends by year (original)

Year	*Campylobacter* spp.	*Salmonella* spp.	*Listeria monocytogenes*
2008	5.2788	5.1761	3.1761
2009	5.2900	5.1614	3.2041
2010	5.3010	5.1461	3.2304
2011	5.3222	5.1303	3.2553
2012	5.3424	5.1139	3.2788
2013	5.3617	5.0969	3.3010
2014	5.3802	5.0792	3.3222
2015	5.3711	5.0682	3.3010
2016	5.3617	5.0607	3.3118
2017	5.3522	5.0531	3.3222
2018	5.3424	5.0414	3.3324
2019	5.3324	5.0334	3.3424
2020	5.3222	5.0253	3.3522
2021	5.3118	5.0170	3.3617
2022	5.3010	5.0086	3.3711

Converting to logarithmic scale allows for better visualization of trends across pathogens with significantly different case numbers.

Analysis of logarithmic trends

Campylobacter spp. shows logarithmic values between 5.28-5.38, with a peak in 2014 (5.3802) followed by a gradual decline to 5.3010 in 2022. This represents a relatively small but consistent downward trend on the logarithmic scale.

Salmonella spp. demonstrates a consistent downward trend from 5.1761 in 2008 to 5.0086 in 2022. This represents approximately a 0.17 log reduction over the 15-year period, indicating a substantial and persistent decline in cases.

Listeria monocytogenes shows the most significant relative increase, with logarithmic values rising from 3.1761 in 2008 to 3.3711 in 2022. This represents approximately a 0.20 log increase, which is particularly noteworthy given its lower baseline incidence.

On the logarithmic scale, the difference between *Campylobacter/Salmonella* and *Listeria* becomes much more apparent. While *Campylobacter* and *Salmonella* remain in the 5.0-5.4 range, *Listeria* values are consistently in the 3.1-3.4 range, highlighting the approximately 100-fold difference in case numbers.

While the absolute number changes for *Listeria* appear small (from 1,500 to 2,350 cases), the relative increase (approximately 57%) is actually more substantial than might be initially perceived.

The logarithmic representation provides a clearer picture of the relative changes over time, particularly for *Listeria monocytogenes*, whose trend might be visually obscured in linear graphs due to its significantly lower case numbers compared to *Campylobacter* and *Salmonella*.

Campylobacteriosis was the most frequent zoonotic illness in the EU in 2016 and 2017, maintaining its status as a leading cause of foodborne illness. The previously observed upward tendency of cases, beginning in 2008, stabilized from 2012 to 2017. This persistence called for reinforced food safety measures and continued public health vigilance.

Despite this stabilization, *Campylobacter* remained a significant pathogen of concern, particularly due to its high prevalence in poultry, which was a primary source of infection. The findings suggest that while the incidence of *Campylobacter* infections may have plateaued, ongoing efforts to monitor and control this pathogen in the food supply are crucial to prevent potential outbreaks.

In 2018, campylobacteriosis and salmonellosis ranked first and second, respectively, among the zoonoses most frequently affecting humans in Europe. The trend of confirmed human cases for both diseases remained stable during the assessment period. Campylobacteriosis

continued to be a serious concern, particularly regarding its association with poultry products. This stability suggests that while control measures may be effective in managing outbreaks, the persistent prevalence of these pathogens in the food supply remains a concern.

In 2020, campylobacteriosis was the most frequently reported zoonosis in the EU, with approximately 121,000 recorded cases, marking a significant decrease from over 220,000 cases in 2019.

Salmonellosis followed closely with 52,702 reported cases, down from approximately 88,000 the previous year. The overall decline in zoonotic outbreaks was drastic, with a notable 47% decrease attributed to the COVID-19 pandemic. Factors contributing to this decline included changes in health-seeking behavior, travel restrictions, restaurant closures, quarantine measures, and the implementation of health protocols such as mask-wearing, physical distancing, and enhanced hand hygiene.

In 2022, campylobacteriosis and salmonellosis remained at the top, both being the most frequent zoonotic illnesses, with case numbers stable compared to 2021. Campylobacteriosis continues to be a significant concern, as it is often associated with poultry and contaminated water sources. Salmonellosis, likewise, persists as a major public health issue, particularly due to its association with poultry and egg products. The need for intensified food safety measures and monitoring systems to mitigate the infections associated with *Campylobacter* in the food industry emphasizes its role as a leading cause of foodborne illness in Europe.

Salmonellosis trends and compliance

In contrast to the rising cases of campylobacteriosis, the EU has observed a declining trend in confirmed human salmonellosis cases between 2008-2017. However, there has been a resurgence of *Salmonella enteritidis* cases, while levels of *S. stanley* have remained elevated compared to the 2011-2012 period. While most MSs registered a reduction in *Salmonella* in poultry, some struggled to achieve these goals in relation to laying hens, highlighting disparities in *Salmonella* control efforts within the EU.

In 2018, the number of human salmonellosis cases attributed to *S. enteritidis* mirrored levels observed in 2017, highlighting a persistent challenge in controlling this serovar. The prevalence of targeted *Salmonella* serovars in various poultry categories exhibited a decreasing trend in the EU; however, this trend plateaued in breeding turkeys, indicating a need for renewed focus on this area.

Notably, the number of salmonellosis cases attributed to *S. enteritidis* reported in the EU in 2019 was consistent with the levels observed in 2017-2018, indicating ongoing challenges in addressing this particular serovar.

Salmonella remained in 2020 the most prevalent agent causing FBOs, accounting for approximately 23% of the total incidents, with eggs, egg products, and pig meat identified as primary sources of salmonellosis outbreaks (EFSA, 2021).

In 2021, among the reporting MSs and the UK, nineteen MSs successfully met all conditions for reducing the prevalence of *Salmonella* serovars in poultry populations. This achievement highlights the effectiveness of national control programs and interventions aimed at reducing *Salmonella* prevalence in the food supply chain. However, it also emphasizes the need for continued vigilance and enforcement of food safety regulations, as outbreaks can still occur.

Notably, samples collected by competent authorities from animal carcasses for *Salmonella* quantification and from broiler carcasses for *Campylobacter* exhibited higher positivity rates than those from internal checks conducted by food business operators. This discrepancy suggests that there may be gaps in monitoring and compliance within the food industry, necessitating enhanced oversight and more stringent inspection protocols to ensure food safety (EFSA, 2022).

Continued vigilance and targeted interventions are needed to address the varying *Salmonella* prevalence across different MSs and poultry categories.

Listeriosis and other emerging zoonoses

Listeriosis cases were on the rise between 2008 and 2014, with the report underscoring the limited occurrences of *Listeria* spp. exceeding food safety thresholds in RTE (ready-to-eat)

foods established within the EU. This trend highlights the need for ongoing vigilance in food safety practices, particularly concerning vulnerable populations (EFSA, 2015).

However, the number of listeriosis cases stabilized in 2015. *Listeria monocytogenes* was rarely found to exceed thresholds in RTE foods, reflecting the application of effective control measures. The need for ongoing vigilance and monitoring remains, especially given the pathogen's potential to cause severe illness in vulnerable populations (EFSA, 2016).

Confirmed cases of listeriosis continued to rise in 2016 and 2017, reflecting an ongoing public health concern. Despite the increase in cases, *L. monocytogenes* rarely exceeded thresholds in RTE foods, suggesting that existing food safety regulations and monitoring practices are effective in mitigating risks associated with this pathogen. This trend underscores the potential risks associated with listeriosis, particularly for vulnerable individuals (EFSA, 2017; EFSA, 2018).

The listeriosis cases reported in 2018 continued to rise, despite *L. monocytogenes* rarely exceeding limits in RTE foods. This trend suggests that while regulatory measures are effective in controlling *Listeria* levels in food products, the increasing incidence of listeriosis cases indicates possible gaps in consumer awareness, food handling practices, or the presence of *Listeria* in environments not adequately addressed by current food safety measures (EFSA, 2019).

The fluctuation in listeriosis cases in the EU remained steady from 2015 to 2019, following a prolonged period of increase. *Listeria monocytogenes* rarely exceeded limits in RTE foods, indicating that existing food safety regulations and monitoring practices are effective in controlling this pathogen. However, the steady trend in listeriosis cases suggests that further efforts are needed to ensure the safety of vulnerable populations at higher risk of severe illness (EFSA, 2020).

There was a descending trend in yersiniosis cases from 2008 to 2012, with *Yersinia* commonly detected in pork and related products.

Verocytotoxigenic *Escherichia coli* (VTEC) infections showed a slight decrease in 2014 compared to 2013, with notable reports linking VTEC to both food and animal sources. The trend in confirmed yersiniosis cases declined in 2015, with most cases predominantly linked to *Yersinia* in pork. These trends highlight the importance of monitoring food sources associated with yersiniosis to prevent outbreaks (EFSA, 2015).

The previously observed tendency of yersiniosis cases since 2008 stabilized during the period from 2012 to 2017. This stabilization reflects the plateau observed in Shiga toxin-producing *Escherichia coli* (STEC) infections, which remained consistent with previous years with frequent reports linking STEC to ruminant meat products. The persistence of STEC in the food supply underscores the need for ongoing surveillance and consumer education regarding safe food handling practices.

Yersinia spp. and STEC continue to present public health challenges, and monitoring efforts must remain robust to prevent potential outbreaks linked to these pathogens.

STEC zoonotic infections emerged as the third most commonly reported zoonosis in the EU, with a notable increase from 2014 to 2019. This rise in STEC infections underscores the importance of monitoring ruminant meat products, which are frequently associated with these pathogens. Yersiniosis ranked fourth among the most frequent zoonotic diseases in 2018, maintaining a consistent trend throughout the assessment period. Continued vigilance is necessary to manage these infections and prevent potential outbreaks linked to food sources (EFSA, 2019; EFSA, 2020).

In 2020, zoonotic diseases reported included yersiniosis (over 5,500 cases) and infections with STEC (over 4,400 cases). Listeriosis ranked fifth with over 1,800 cases, predominantly affecting vulnerable individuals. Notably, listeriosis and West Nile virus infections provoked the highest case fatality and hospitalization rates, with cases concentrated in Greece, Spain, and Italy.

Yersiniosis ranked as the third most frequently reported zoonosis in humans in 2022. The persistence of yersiniosis as a notable zoonotic disease underscores the importance of surveillance and control measures, particularly in relation to food sources commonly linked to infection, such as pork and unpasteurized milk.

Infections caused by Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes* followed yersiniosis in the ranking of reported zoonoses. The presence of STEC in food products, particularly undercooked beef and contaminated produce, remains a significant public health concern. *Listeria monocytogenes* infections were noteworthy due to their association with RTE foods and their potential severity in vulnerable individuals.

Over 5,250 foodborne outbreaks (FBOs) were counted in 2014. The waterborne outbreaks (WBOs) were predominantly attributed to viral agents, followed by bacterial pathogens such as *Salmonella*, bacterial toxins, and *Campylobacter*. Approximately 29% of outbreaks involved unidentified causative agents, highlighting gaps in current surveillance and reporting systems. Key food sources implicated in documented outbreaks included eggs and egg products, mixed foods, and shellfish, reiterating the importance of targeted interventions in these areas.

Bacterial foodborne zoonotic pathogens were found to play a substantial role in human diarrheal diseases, accounting for 64% of cases reported, followed by viral infections at 24% and protozoan infections at 12%. This distribution emphasizes the critical need for comprehensive strategies to address both bacterial and viral zoonotic threats (EFSA, 2015).

In 2015, a total of 4,362 FBOs and WBOs were documented. Bacterial pathogens were identified as the primary cause, followed by bacterial toxins as the secondary cause, viruses as the third, and parasites as the least common. Alarmingly, the responsible agent is still unidentified in over 33% outbreaks, indicating potential gaps in surveillance and reporting systems. *Salmonella* in eggs was recognized as the most dangerous FBOs agent, consistent with trends observed in previous years (EFSA, 2016).

In 2016, a total of 4,786 FBOs and WBOs were recorded. *Salmonella* was the most frequent pathogen, with *S. enteritidis* particularly prominent (EFSA, 2017).

In 2017, over 5,000 FBOs and WBOs were found. *Salmonella* was identified as the most frequently detected causative agent, particularly *S. enteritidis*. Other bacterial pathogens, bacterial toxins, and viruses were also found.

Alarmingly, the causative agent remained undetermined in over 37.5% of FBOs and WBOs cases, highlighting gaps in surveillance and identification processes. Moreover, *Salmonella* was identified as posing the highest risk for foodborne infections after the ingestion of eggs, meat, or mixtures, underscoring the need for targeted interventions and consumer education regarding safe food handling practices (EFSA, 2018).

Over 5,100 FBOs and WBOs were documented in 2018 and *Salmonella* was declared the most prevalent pathogen. Notably, *S. enteritidis* accounted for approximately one-fifth of these outbreaks, highlighting the continued risk associated with this serovar. *Salmonella* in eggs and egg products was the most dangerous FBOs pathogen, necessitating targeted intervention strategies to reduce the incidence of foodborne illness related to these products.

Additionally, in 2018 was noted a significant surge in West Nile virus infections, illustrating the evolving landscape of zoonotic threats in Europe. This increase raises concerns about vector-borne diseases and the need for integrated surveillance and control measures to address emerging zoonotic risks (EFSA, 2019).

In 2019, over 5,170 FBOs were documented, with *Salmonella* as the most frequently detected pathogen. The FBOs attributed to *S. enteritidis* witnessed a decrease, highlighting the impact of targeted control measures. Interestingly, norovirus in seafood emerged as the cause for the highest number of well-supported FBOs, marking a shift in focus for food safety efforts. This emerging trend necessitates enhanced monitoring and control measures for seafood products to mitigate the risks associated with norovirus infections (EFSA, 2020).

In 2021, campylobacteriosis and salmonellosis were reported as the most related to human gastrointestinal infections, with nearly 130,000 and 60,500 cases, respectively. This represented an increase of approximately 7,000 cases for both illnesses compared to 2020. Factors such as the UK's withdrawal from the EU and ongoing COVID-19 lockdowns affected data collection and analysis.

National *Salmonella* control programs in poultry aimed to reduce human illnesses caused by specific *Salmonella* serovars. In 2021, certain countries met targets for reducing *Salmonella* presence in poultry. Stable improvements were observed within the EU. Food samples generally showed low *Salmonella* presence, with slightly higher rates in meat, meat products, and spices.

Various zoonotic diseases, including yersiniosis, STEC infections, and listeriosis, posed significant health risks in 2021. Infections caused by both *Listeria monocytogenes* and West Nile virus were associated with high hospitalization and mortality rates. Surveillance also covered diseases such as brucellosis, Q fever, and toxoplasmosis.

A total of 4,005 FBOs were reported in 2021, marking a 30% increase from 2020 and leading to a total of 32,543. These figures closely aligned with levels observed during the pre-pandemic period of 2017-2019, suggesting a gradual return to normal food consumption behaviors in restaurants and food service settings, which could facilitate the transmission of foodborne pathogens.

S. enteritidis, was the most frequent FBOs agent. Among various food categories, eggs, egg derivatives, and mixed foods were identified as the most common infection sources, with a significant increase in outbreaks related to plant-based foods compared to previous years. Identifying and addressing the sources of *Salmonella* in food products, particularly eggs and poultry, is vital to reduce the incidence of foodborne illness.

S. The 2020 and 2021 EFSA and ECDC reveal critical trends in zoonotic diseases, highlighting a significant impact of the Coronavirus 2019 pandemic on disease reporting and public health behaviors. While the decline in reported cases in 2020 can be attributed to pandemic-related factors, the increase in cases in 2021 suggests a return to pre-pandemic food consumption patterns. The persistence of campylobacteriosis and salmonellosis as leading zoonotic diseases underscores the need for ongoing surveillance and effective control measures. The introduction of interactive communication tools by EFSA enhances public awareness and supports data-driven decision-making in food safety.

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Severity of zoonotic diseases

In 2022, among the reported zoonotic diseases, infections caused by *L. monocytogenes* and West Nile virus provoked the highest hospitalization and mortality rates. The emergence of illnesses caused by the West Nile virus highlights the need for continued surveillance and vector control efforts (EFSA, 2015).

Listeriosis poses significant risks due to its potential for severe outcomes, including meningitis and septicemia, particularly among high-risk individuals.

The 2014 report from the EFSA and the ECDC presents the findings of disease surveillance conducted in 28 MSs and 4 non-MSs.

Campylobacteriosis emerged as the most frequently reported zoonosis, showing an upward trend within the EU since 2008. The prevalence of *Campylobacter* in broiler meat remained high.

Contrastingly, after 2008, the EU observed a reduction in confirmed human salmonellosis cases, accompanied by an uptick in instances of *S. enteritidis*. *S. stanley* cases persisted at elevated levels compared to 2011-2012. Most MSs achieved the reduction of *Salmonella* in poultry, although there was an increase in *S. infantis* isolates at the EU level.

Moreover, human listeriosis cases have continued to rise since 2008, with limited instances of *Listeria* exceeding safety thresholds in RTE foods. After 2008, a descending trend in confirmed yersiniosis cases was observed, with *Yersinia* frequently detected in pork and related food. VTEC infections decreased in 2014 compared to 2013.

A number of 5,250 FBOs and WBOs were documented, predominantly attributed to viruses, followed by *Salmonella*, bacterial toxins, *Campylobacter*, and unidentified causative agents in approximately 29% of total FBOs. The food sources in well-documented FBOs included eggs, egg products, mixed food, crustaceans, molluscs, and their derivatives.

Bacterial food-borne zoonotic pathogens played a significant role in causing human diarrheal diseases, accounting for 64% of cases,

followed by viral infections at 24% and protozoan infections at 12%.

A report published by EFSA and the ECDC highlights the findings of monitoring activities conducted in 2015 across 32 European countries (EFSA, 2015).

Campylobacteriosis emerged as the most frequently reported zoonosis, with a continuous rise in cases within the EU since 2008. Notably, *Campylobacter* prevalence has remained consistently elevated in broiler meat. Conversely, the reduction of salmonellosis incidents in the EU persisted, although there was a rise in human *Salmonella enteritidis* instances. The majority of MSs achieved their goals for poultry, with a surge in *S. enteritidis* isolates. Regarding food safety, the EU observed low levels of *Salmonella* non-compliance in poultry prepared meat.

In 2015, *Listeria monocytogenes* rarely exceeded limits in RTE foods.

The decline in yersiniosis cases in the EU persisted, with the bacteria predominantly linked to findings in pork and its derivatives. STEC levels remained consistent with those observed in 2014 and were frequently associated with ruminant products.

A number of 4,360 FBOs, encompassing WBOs, were documented, with bacteria as the primary identified causative agents, followed by bacterial toxins, viruses, and parasites. In 33.5% of FBOs cases, the causative agent remained unidentified. Notably, *Salmonella* in eggs remained the most dangerous FBOs agent (EFSA, 2016).

The outcomes of zoonoses monitoring activities conducted by EFSA and ECDC in 2016 across European countries (n = 37), showed that campylobacteriosis remained the most frequently reported zoonosis.

The increase in confirmed human cases within the EU since 2008 stabilized during the period of 2012-2016. Notably, the prevalence of *Campylobacter* remained high in broiler meat. The decline in salmonellosis cases in humans in the EU since 2008 remained steady during the years 2012-2016, accompanied by an increase in human *S. enteritidis* cases.

Generally, MSs achieved the reduction objectives for poultry, except for laying hens in 5 MSs. In primary production, the prevalence of target *Salmonella* serovars in breeding hens, broilers, and turkeys remained generally stable compared to previous years, while the prevalence of *S. enteritidis* in laying hens showed a notable increase. Regarding food safety, levels of *Salmonella* non-compliance in minced meat and poultry meat preparations were low.

The number of listeriosis cases increased in 2016, despite *Listeria* rarely exceeding safety limits in RTE foods. The decreasing trend in confirmed yersiniosis cases in the EU since 2008 stabilized during 2012-2016.

Approximately 4,800 FBOs and WBOs cases were recorded, with *Salmonella* being the most frequently identified causative agent, particularly *S. enteritidis* (EFSA, 2017).

The findings of zoonoses monitoring activities conducted by EFSA and ECDC in 2017 across 37 EU countries showed that the most commonly reported zoonosis was campylobacteriosis, with the previously increasing trend since 2008 stabilizing between 2013 and 2017.

The downward pattern in documented human *Salmonella* infections within the European Union since 2008 paused between 2013 and 2017. This pause was accompanied by an increase in human *Salmonella enteritidis* infections, largely due to one MSs beginning the reporting of serotype data. Sixteen nations successfully met all goals for poultry *Salmonella* reduction, while twelve nations failed to meet at least one goal.

The overall rate of specific *Salmonella* serotypes in various poultry types within the EU either fell or remained the same compared to 2016, with a minor rise observed in breeding turkeys. Discrepancies in *Salmonella* results for pig carcasses and serovars in poultry were noted between regulatory bodies and food industry operators.

The number of reported human listeriosis cases rose in 2017, though *Listeria* rarely exceeded the safety limits in RTE foods. The downward pattern in documented yersiniosis cases within the EU since 2008 paused between 2013 and 2017, and the rate of confirmed shiga toxin-producing *E. coli* infections in humans remained consistent. Nearly 5,080 FBOs and WBOs were reported, with *Salmonella* being the most common cause, particularly *S. enteritidis*, followed by other bacteria,

bacterial toxins, and viruses. The cause was unknown in 37.6% of all incidents, with *Salmonella* in eggs and meat/blends posing the greatest risk of foodborne infections (EFSA, 2018).

The collaborative effort between the EFSA and the ECDC revealed the results of animal-borne disease surveillance activities conducted in 2018 across 36 European nations, including 28 MSs and 8 non-MSs.

Campylobacteriosis and salmonellosis were identified as the primary and secondary most frequently reported zoonotic diseases in humans, respectively. The trend of confirmed human cases for these two diseases within the European Union (EU) remained stable during the assessment between 2014 and 2018.

The percentage of human *Salmonella* infections caused by *Salmonella enteritidis* in 2018 matched the levels seen in 2017.

Among the 27 reporting nations, 16 achieved all *Salmonella* reduction goals for chickens and turkeys, while 11 fell short of meeting at least one goal. The overall rate of specific *Salmonella* serotypes in various poultry types within the EU showed a downward pattern in recent years but leveled off in breeding turkeys.

Significantly, results for *Salmonella* from regulatory bodies for pig carcasses and poultry sampled through National Control Programs showed higher rates of positive tests compared to those from food industry operators.

Shiga toxin-producing *E. coli* infections in humans became the third most commonly reported animal-borne disease in the EU, with a significant increase from 2014 to 2018.

Yersiniosis was the fourth most frequently reported zoonotic illness in humans in 2018, maintaining a consistent pattern between 2014 and 2018. The count of confirmed listeriosis cases reported in 2018 continued to climb, even though *Listeria* rarely surpassed the safety limit in RTE foods.

Nearly 5,150 FBOs and WBOs were recorded, with *Salmonella* recognized as the most widespread cause, particularly *S. enteritidis*, accounting for one-fifth of the incidents. *Salmonella* in eggs and egg-based items were identified as the highest risk combination of cause and food. Furthermore, a substantial increase in human West Nile virus infections was noted in 2023 (EFSA, 2019).

The combined report from the EFSA and the ECDC discloses the results of animal-borne disease surveillance activities carried out in 2019 across 36 European nations.

Campylobacteriosis and salmonellosis were recognized as the most frequently reported animal-borne illnesses in humans, with the trend of confirmed human cases for these conditions in the European Union (EU) showing a consistent pattern during the period of 2015-2019.

The proportion of human salmonellosis cases attributed to *Salmonella enteritidis* within the EU aligned with the levels observed between 2017 and 2018.

Of the 26 nations providing information on *Salmonella* control programs in poultry, 18 met the reduction goals, while eight did not achieve at least one target. The overall rate of flocks testing positive for targeted *Salmonella* serovars in the EU remained consistent since 2015 for breeding hens, laying hens, broilers, and fattening turkeys, with some variations noted for breeding turkey groups.

Significant differences were observed in *Salmonella* results from regulatory bodies for pig carcasses and poultry sampled via national control programs compared to those from food industry operators.

Shiga toxin-producing *E. coli* infections became the third most reported animal-borne disease in humans and showed an increase from 2015 to 2019.

Yersiniosis remained the fourth most commonly reported zoonotic disease in humans in 2019, maintaining a consistent pattern between 2015 and 2019. The EU pattern for confirmed listeriosis cases remained stable during 2015-2019 after a long period of increase. *Listeria* rarely exceeded the safety limit in RTE foods.

Nearly 5,180 food-related incidents were recorded, with *Salmonella* remaining the most frequently identified cause, although the number of incidents attributed to *S. enteritidis* showed a reduction.

Remarkably, norovirus in seafood was identified as the combination of cause and food leading to the greatest number of confirmed incidents (EFSA, 2020).

In 2020, campylobacteriosis was identified as the most prevalent animal-borne disease in the

European Union, with nearly 121,000 cases documented. This represented a substantial drop from the prior year when over 220,000 cases were reported.

Closely following was salmonellosis, impacting 52,700 individuals compared to 88,000 in 2019. The reduction in reported animal-borne illnesses and food-related incidents was severe, with a significant 47% drop.

The One Health zoonosis report, released by the EFSA and the ECDC, highlighted the impact of the COVID-19 pandemic. Experts attributed the registered decrease to changes in health-seeking behavior, travel restrictions, closures of restaurants, quarantine measures, and the implementation of various health protocols such as mask-wearing, physical distancing, and enhanced hand hygiene.

Yersiniosis (5,668 cases) and infections caused by Shiga toxin-producing *E. coli* (4,446 cases) were the subsequent most frequently reported illnesses. Listeriosis was the fifth most reported animal-borne disease with 1,876 cases, primarily affecting individuals over the age of 64.

Listeriosis and West Nile virus infections had the highest death and hospitalization rates, with a significant concentration of West Nile virus cases reported in Greece, Spain, and Italy. *Salmonella* remained the most widespread cause of food-related incidents, accounting for approximately 23% of the total occurrences. Eggs, egg-based items, and pig meat were identified as the main sources of salmonellosis incidents (EFSA, 2021).

EFSA launched two engaging communication resources - a narrative map and a data display - to improve the understanding of food-related incidents. The narrative map provides a detailed overview of food-related incidents, causative agents, and implicated food sources.

Conversely, the data display acts as a platform for users to explore and examine extensive data on food-related incidents gathered by EFSA from EU member nations and other reporting countries since 2015. These tools aim to improve transparency, facilitate data-driven decision-making, and enhance public awareness of food safety within Europe.

Each year, the ECDC and the EFSA work together to collect information on animal-borne illnesses from member countries and other European areas. This comprehensive dataset includes information on zoonotic microorganisms found in various sources such as food, feed, animals (both livestock and wildlife), and humans. By analyzing this data, ECDC and EFSA produce a detailed report highlighting the prevalence of zoonotic agents across different sources and trends observed in humans, food products, and animals.

By employing descriptive statistics and pattern analyses, they take into account elements like data quality and collection methods to offer insights for animal health and food safety agencies as well as decision-makers. The objective is to steer strategic planning to lessen animal-borne illness impact and decrease human cases. This data-focused procedure adheres to a "One Health" framework.

In 2021, campylobacteriosis and salmonellosis were the most frequently documented animal-borne digestive illnesses in humans, with 127,840 and 60,050 cases, respectively.

There was an increase of approximately 7,000 cases for both illnesses compared to the previous year. Factors such as the UK's withdrawal from the EU and COVID-19 lockdowns affected data collection and analysis. National *Salmonella* control programs in poultry aim to reduce human illnesses caused by specific *Salmonella* serovars.

In 2021, several nations met goals for decreasing *Salmonella* rates in poultry, with consistent patterns observed in the EU. Food tests showed minimal *Salmonella* presence overall, with modestly higher rates in meat, meat-based items, and seasonings.

Various animal-borne illnesses, including yersiniosis, STEC infections, and listeriosis, presented substantial health hazards in 2021. *Listeria monocytogenes* and West Nile virus infections were the most serious, resulting in high hospitalization and death rates. Surveillance also included illnesses like brucellosis, Q fever, and toxoplasmosis.

In 2021, 27 member states and the United Kingdom (Northern Ireland) documented 4,000 food-related incidents, which represented a 30% rise from 2020, leading to a total of 32,543 human cases, showing a 62.6% increase. These figures closely matched the

levels seen during the period of 2017-2019 before the start of the COVID-19 pandemic.
This implies a steady return to pre-pandemic food consumption habits in 2021, especially in eateries, cafeterias, and other food service locations that could contribute to the spread of food-related pathogens.
Salmonella, notably *S. enteritidis*, continued to be the most common agent causing FBOs.
Among the various food types, eggs and egg-based items and combination foods were identified as the most frequent causes in these incidents, as indicated by the data supplied by the member countries. Occurrences related to the consumption of items in the greens and juices and other related items category showed a marked increase in 2021 compared to previous years (EFSA, 2022).
The detailed report from the EFSA and the ECDC summarizes the results from animal-borne disease monitoring and surveillance efforts conducted in 2022 across 27 member nations, the United Kingdom (Northern Ireland), and 11 countries outside the EU. The report examines key statistics regarding animal-borne diseases and causative agents in humans, food, animals, and feed, offering historical interpretations.
In 2022, campylobacteriosis and salmonellosis were identified as the top two most documented animal-borne illnesses in humans, with the case count remaining consistent compared to 2021. Nineteen member nations and the United Kingdom (Northern Ireland) successfully achieved all established goals for lowering the rate of *Salmonella* for relevant serotypes in poultry populations.
Remarkably, specimens gathered by regulatory agencies from animal carcasses for *Salmonella* and from broiler carcasses for *Campylobacter* measurement showed greater rates of positive tests compared to those from in-house inspections. Yersiniosis (Figure 2) was the third most commonly documented animal-borne disease in humans, followed by STEC and *Listeria monocytogenes* infections. Illnesses resulting from *L. monocytogenes* and West Nile virus were identified as the most serious animal-borne diseases, linked to the greatest rates of hospital admissions and death.

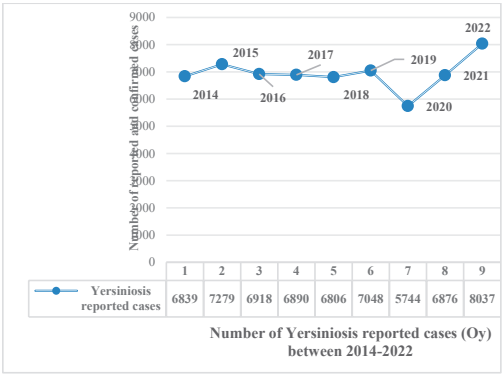


Figure 2. Yersiniosis reported number of cases (Oy) between 2014 and 2022 (Ox) - original

In 2022, there was a significant rise in both animal-borne illnesses and food-related incidents, leading to a thorough examination of documented cases, associated hospital admissions, and deaths. A concerning jump of over 600% in locally acquired cases of human West Nile virus infection, a disease carried by mosquitoes, was documented in 2022 compared to 2021 (Figure 3).

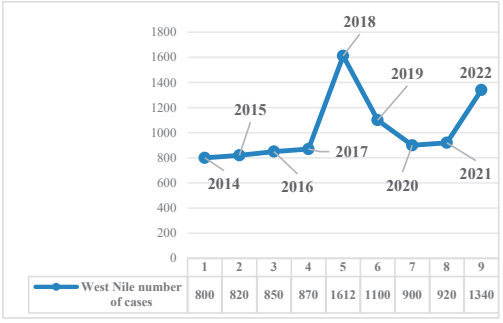


Figure 3. West Nile number of cases (Oy) between 2014 and 2022 (Ox) - original

The EU saw a surge in reported food-related incidents, cases, hospital admissions, and deaths in 2022 compared to the prior year, with the greatest count of incident-related deaths reported in the past ten years, mainly attributed to *Listeria monocytogenes* and to a lesser degree, *Salmonella*. Particularly *S. enteritidis*, remained the primary cause in food-related incidents, with Norovirus and other caliciviruses identified as the causes linked to the greatest number of human infection in incidents.

Furthermore, the report provides current information on trichinellosis, rabies, brucellosis, toxoplasmosis, Q fever, echinococcosis, tularemia and tuberculosis (EFSA, 2023).

The One Health animal-borne diseases report released by EFSA and ECDC explains the animal-borne disease situation in 2022. Salmonellosis and campylobacteriosis remained the most documented animal-borne illnesses in humans within the EU for the eighth consecutive year.

Despite their common occurrence, the case counts for these illnesses stayed below the levels observed in the pre-pandemic years of 2018-2019.

In the case of campylobacteriosis, which is the most commonly documented animal-borne illness, the case count remained consistent in 2022, totaling 137,100 cases.

Chicken meat was identified as the main source of these infections, emphasizing the importance of food safety precautions in poultry production (Figure 4).

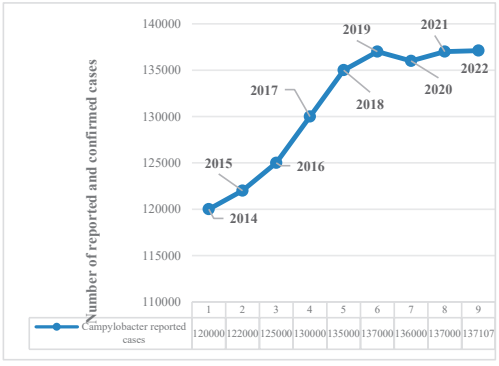


Figure 4. Campylobacteriosis number of cases (Oy) in the EU between 2014 and 2022 (Ox) - original

Salmonellosis, the second most frequently documented animal-borne illness, recorded 65,200 cases in 2022, a small increase from 2021 when 60,170 cases were documented (Figure 5).

Encouragingly, 19 MSs and the United Kingdom (Northern Ireland) successfully achieved all targets set for reducing *Salmonella* in poultry populations.

This marked achievement signifies significant progress since 2018 when only 14 MSs met these targets, showcasing collective efforts in combating zoonotic diseases and safeguarding public health.

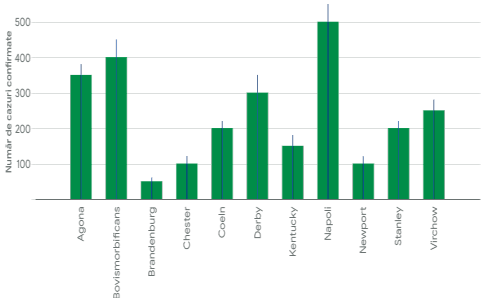


Figure 5. Salmonellosis number (Oy) of cases (5th-15th serovars) in 2022 vs. 2018-2021 average (Ox), in EU/EEA (EFSA, 2023)

ECDC emphasized the importance of sustained vigilance and concerted efforts in reducing the burden of these common foodborne illnesses on public health. Despite the current lower levels of reported cases compared to pre-pandemic times, the impact of these infections necessitates continued attention and actions aimed at further reducing their prevalence in the population.

In 2022, the European Union experienced a significant surge in illnesses caused by the mosquito-borne disease, resulting in the second-highest number of cases on record for the region, with 1,133 instances reported. The peak year for West Nile virus infections remains 2018, with 1,612 cases reported. This surge in cases could be attributed to more conducive climatic conditions supporting increased mosquito activity, facilitating the spread of the virus.

The presence of this specific virus was identified in 431 birds and 166 horses within the EU in a particular year, roughly twice the numbers reported in the year before. Furthermore, the virus spread to areas not previously affected, including parts of France, Germany, and Italy.

The unit focused on biological risks and animal well-being. EFSA emphasized the growing danger of illnesses transmitted by insects due to changes in weather patterns. The agency stressed the need for a comprehensive approach that combines assessments of danger for people and animals to effectively address these new health problems.

In addition to isolated cases of illness, the report looks into investigations of instances where the infection was multiple.

The European Union saw a significant increase in reported cases of foodborne illness, from 4,000 in one year to nearly 5,760 in the following year, returning to levels seen before a global health crisis. This rise in cases led to the highest number of deaths related to foodborne illness in the last ten years, amounting to 64 fatalities.

Listeria monocytogenes was identified as a primary causative agent (Figure 6), with outbreak-associated deaths linked to a diverse array of food items spanning meat, fermented milk products, fish, and greens. The greater use of a detailed genetic mapping method likely increased the accuracy of monitoring and improved the ability to find outbreaks across various nations.

EFSA is launching interactive story maps and dashboards offering insights into *Brucella*, zoonotic tuberculosis, Shiga toxin-producing *E. coli*, enabling data visualization and exploration for stakeholders.

The year 2022 saw an unusually high number of deaths recorded in FBOs reported to the EFSA within the EU over the past decade.

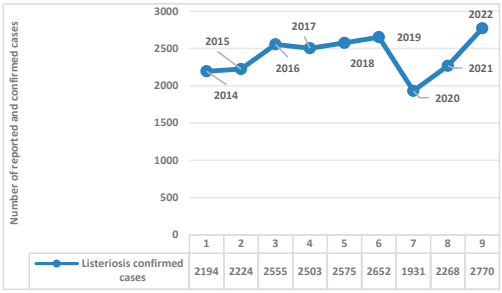


Figure 6. Listeriosis number of cases (Oy) between 2014 and 2022 (Ox)

Streptococcus equi subspecies *zooepidemicus*, a newly recognized etiologic agent, demonstrated the highest mortality rate in 2022. A significant foodborne transmission event, associated with the consumption of cheese produced from unpasteurized milk, resulted in 37 confirmed cases, including five fatalities, highlighting the virulence of this microorganism.

Listeria monocytogenes accounted for a significant portion of these fatalities. This emphasizes the severe health consequences linked to *L. monocytogenes*, particularly impacting vulnerable demographics such as the elderly.

Salmonella species were identified as the dominant etiological agent in foodborne disease outbreaks across the European region, accounting for the largest proportion of cases (over 1,000), representing 17% of FBOs. Furthermore, *Salmonella* species were associated with the highest rate of hospital admissions, accounting for 50.5% of all hospitalizations (Figure 7).

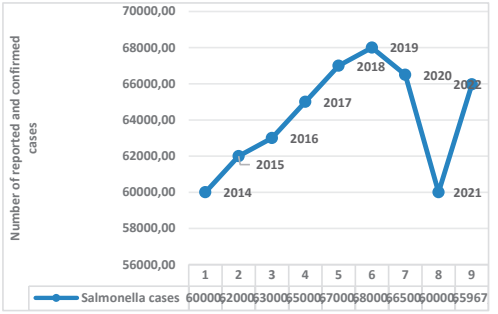


Figure 7. Number of *Salmonella* cases (Oy) by EFSA between 2014 and 2022 (Ox) - original

Norovirus (and other caliciviruses) were the primary etiological agents associated with a high incidence, demonstrating an 11% increase in 2022 (n = 7,300; 15% of outbreak-related cases). These agents were responsible for 12 significant outbreaks involving over 100 cases in multiple countries, with a substantial mean outbreak size of 22 cases.

In 2022, nearly 490 outbreaks with robust epidemiological support were documented, constituting 8.5% of all reported outbreaks. Complex food matrices, including multi-component dishes and miscellaneous food items, were identified as the primary vehicles in the largest proportion of these outbreaks (30%), accounting for the greatest number of associated cases (32% of all cases linked to outbreaks with robust epidemiological support). Notably, for the inaugural time since the initiation of foodborne outbreak surveillance in the EU, the highest number of outbreaks with robust epidemiological support and associated cases occurred in food service establishments, including restaurants, pubs, and mobile food vendors, comprising 30% of all outbreaks with robust epidemiological support. Among the

identified exposure environments, educational facilities, such as schools and kindergartens, recorded a significant burden of cases (1,550 cases; 16% of all cases in outbreaks with robust epidemiological support), emphasizing the importance of these settings in outbreak occurrence.

According to Braam et al. (2021), given the complex interactions between environmental, socio-economic, and anthropogenic factors contributing to the transmission of zoonotic diseases in displacement contexts, it is hypothesized that the movement of displaced populations, particularly in regions reliant on agriculture and livestock, poses significant risks for the introduction and spread of zoonotic pathogens among host communities, domestic animals, and wildlife (Figure 8).

Insufficient resources for livestock in formal relief camps may lead to adaptive strategies by animal owners, such as grazing among host communities' livestock or encroaching on wildlife habitat, potentially elevating the probability of interspecies pathogen dissemination to immunologically vulnerable groups.

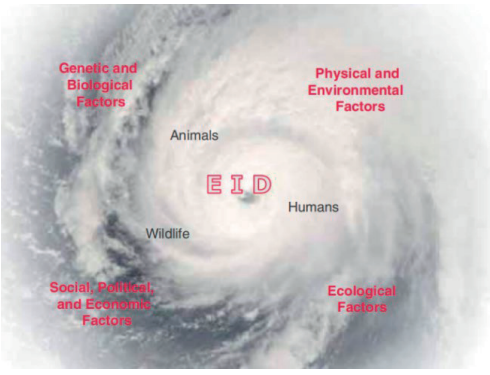


Figure 8. The convergence model of a perfect microbial storm (IOM, 2012)

Throughout the past decade, successive publications have consistently delineated epidemiological patterns and transmission pathways for a spectrum of pathogenic microorganisms, including *Mycobacterium bovis, Trichinella* spp., *Echinococcus* spp., *Toxoplasma gondii, Francisella tularensis, rabies virus, Brucella* spp., *Coxiella burnetii*, and West Nile virus.

Effective interventions for the mitigation of FBOs

Episodes of illness linked to the consumption of tainted foodstuffs continue to represent a substantial challenge to global well-being, resulting in considerable disease burden, fatalities, and financial impact. The World Health Organization (WHO) approximates that 600 million individuals experience adverse health effects annually due to the ingestion of contaminated food, leading to approximately 420,000 deaths. In light of these alarming statistics, it is imperative to adopt effective mitigation strategies to prevent and control foodborne outbreaks. Among the most promising strategies are enhanced traceability, public awareness campaigns, and the implementation of stringent food safety regulations.

Enhanced traceability refers to the ability to track food products throughout the supply chain, from farm to fork. This capability is crucial for quickly identifying and isolating contaminated food products during an outbreak. The implementation of advanced technologies such as barcodes, RFID tags, and blockchain can significantly improve traceability. Employing a chain-linked data structure allows for the establishment of a secure and auditable system for tracking food commodities, affording real-time insight into the distribution chain.

The benefits of enhanced traceability are manifold. Firstly, it enables rapid identification of contamination sources, facilitating swift action to remove affected products from the market. This rapid response is vital in minimizing public exposure to harmful pathogens. Secondly, by improving traceability, food producers and processors can engage in more effective epidemiological investigations, leading to better understanding and management of outbreaks.

Finally, enhanced traceability fosters consumer confidence in food safety. When consumers know that a robust system is in place to track food products, they are more likely to trust the safety of their food supply.

Dissemination initiatives to the general populace are fundamental in conveying knowledge regarding safe food handling procedures. Such programs strive to

communicate the potential hazards related to alimentary pathogen transmission and the significance of appropriate food manipulation, preparation, and preservation. Successful dissemination strategies can utilize diverse modalities, encompassing instructional resources, digital communication platforms, and community-based interventions.

The development of focused educational interventions for vulnerable demographics, including pediatric, geriatric, and immune-deficient populations, is essential. These programs can provide vital information on safe food practices and hygiene. Leveraging social media platforms to disseminate information quickly allows for broader reach and engagement. Additionally, collaborating with local communities, schools, and organizations can amplify food safety messages and encourage safe behaviors.

The impact of public awareness campaigns on food safety is significant. Informed consumers are more likely to adopt safe food handling practices, thereby reducing the risk of foodborne illnesses. Furthermore, increased awareness can lead to greater reporting of foodborne illness cases, aiding health authorities in detecting and responding to outbreaks. By fostering a culture of food safety, these campaigns contribute to overall public health.

Rigorous standards for food safety constitute the regulatory infrastructure required to guarantee the safety of consumables and avert epidemic events. These standards are designed to reduce the probability of pathogen transmission through ingestion and safeguard community well-being. Periodic evaluations of facilities involved in the transformation of edibles, dining establishments, and commercial outlets are indispensable to verify adherence to mandated safety protocols.

Moreover, training and certification programs for food handlers and industry professionals are vital to ensure that they understand and implement effective food safety practices. Risk-based regulations, which prioritize food safety based on scientific assessments of risk, allow for the efficient allocation of resources to areas of higher concern.

Improved adherence to safety benchmarks results in a decreased prevalence of pathogen transmission through ingestion.

The stabilization of listeriosis cases and declining yersiniosis trends in 2015 reflect effective public health measures, yet the ongoing presence of STEC and unidentified outbreak causative agents calls for enhanced surveillance and food safety practices.

While campylobacteriosis remains a significant public health concern, in 2016 the stabilization of trends in salmonellosis, listeriosis, yersiniosis, and STEC infections highlights the complexities of zoonotic disease management. The ongoing challenges presented by *Salmonella*, particularly *S. enteritidis*, and the rising incidence of listeriosis necessitate continued vigilance and improved food safety practices.

Technological advancements within food production and distribution networks have significantly altered the parameters of food safety. A notable trend is the identification of novel or previously uncharacterized pathogenic agents in food matrices, necessitating a re-evaluation of foodborne disease epidemiology.

The increasing incidence of these events underscores the critical need for a comprehensive reassessment of prophylactic strategies. The emergence of previously undocumented microbial species, viral entities, parasitic organisms, and atypical biotoxins from unconventional sources has outpaced the efficacy of existing preventative measures against foodborne disease outbreaks.

The effects on population health and economic burdens, are modulated by a complex interplay of variables influencing the evolution of foodborne pathogens and the emergence of infectious diseases. These variables include alterations in human demographics and behavioral patterns, particularly dietary practices, as well as extreme climatic events, advancements in high-throughput pathogen detection methodologies, microbial adaptation mechanisms, evolving agricultural practices, and transformative shifts within the food processing industry.

Foodborne illnesses constitute a substantial global threat to public health and economic stability. Accurate epidemiological data on foodborne zoonotic diseases are paramount for evidence-based decision-making regarding resource allocation for food safety, regulatory frameworks, and intervention strategies.

Veterinary practitioners occupy a critical position in the initiation of preventive and control measures, commencing at the primary production level through the implementation of Good Practices and biosecurity protocols. Furthermore, the application of the Hazard Analysis and Critical Control Points (HACCP) system across all stages of production and processing is indispensable for mitigating the incidence of foodborne diseases.

The escalating challenge of antimicrobial resistance necessitates the prudent utilization of antimicrobial agents in both human and veterinary medicine. A "One Health" paradigm facilitates a comprehensive understanding of disease epidemiology and enables the effective implementation of control strategies. The adoption of a holistic perspective encompassing all facets of foodborne illnesses is increasingly crucial for safeguarding public health and well-being.

Between 2008 and 2022, *Salmonella* and *Campylobacter* accounted for 62–78% of all reported zoonotic infections in the EU. Salmonella alone triggered 82% of foodborne outbreaks linked to known pathogens, primarily through eggs and poultry products. Despite 19 EU states meeting poultry Salmonella reduction targets, contamination rates in animal carcasses varied widely (4–28%) depending on national testing protocols. Campylobacter infections, though less frequently implicated in outbreaks, represented 65% of all zoonotic cases in 2022, with poultry meat identified as the primary transmission vector.

Listeria monocytogenes caused the highest hospitalization rate (96.1%) among foodborne pathogens, with a case fatality rate of 13.2% in 2022. Its persistence in ready-to-eat foods, such as smoked fish and soft cheeses, exacerbated vulnerabilities in immunocompromised populations. Meanwhile, West Nile virus infections, though not strictly foodborne, surged due to climate-driven expansion of mosquito habitats, recording a 17.9% case fatality rate in 2022.

Surveillance gaps and underreporting

Passive surveillance systems underestimated true disease incidence by 5- to 20-fold, with variability stemming from differences in healthcare access, laboratory practices, and reporting frameworks across MSs. For instance, only 12% of *Campylobacter* cases were laboratory-confirmed, compared to 89% for *Salmonella* (Boqvist et al., 2018).

Emerging threats and climate-driven risks

West Nile virus cases in the EU increased from 74 in 2018 to 1,323 in 2022, with geographic spread into previously unaffected regions like northern Germany and the Netherlands. Rising temperatures and altered precipitation patterns extended the seasonal activity of *Culex* mosquitoes, while migratory bird routes facilitated viral dissemination.

Antimicrobial resistance (AMR) in zoonotic pathogens

Resistance to fluoroquinolones in *Campylobacter jejuni* isolates reached 60% in 2022, limiting treatment options for severe cases. Multidrug-resistant *Salmonella* strains (e.g., S. Kentucky ST198) showed resistance to ciprofloxacin and third-generation cephalosporins, linked to prophylactic antibiotic use in poultry farming.

Vulnerabilities in fresh produce supply chains

Contaminated leafy greens and berries caused 18% of outbreaks in 2022, often traced to irrigation water contaminated by livestock runoff. Decentralized production networks complicated the trace-back efforts, delaying outbreak containment.

Geospatial data in epidemiological surveillance

Geospatial tools enable the integration of environmental, climatic, and demographic data to predict pathogen hotspots. For example, EFSA's review highlighted GIS applications correlating West Nile virus outbreaks with wetland distribution, avian migration corridors, and land surface temperatures. In Italy, spatial models identified provinces with ≥25°C summer temperatures and ≥60% irrigation coverage as high-risk zones, guiding preemptive mosquito control.

A 2021 geospatial analysis overlayed *Salmonella* prevalence data from 3,000 poultry farms with variables like proximity to water sources, livestock density, and slaughterhouse

locations. Farms within 2 km of water bodies had 3.2× higher contamination odds, prompting revised EU regulations on poultry housing siting.

Real-time outbreak source tracking

The EU's One Health sequencing database, launched in 2022, integrates whole-genome sequencing (WGS) data from human, animal, and food isolates with geospatial metadata. During a 2023 *Listeria* outbreak linked to smoked salmon, phylogeographic models traced the origin to a coastal processing plant in Poland, leveraging WGS clusters and transportation route mapping.

ENDIG or a pan-european surveillance visualization

The ENDIG platform aggregates notifiable disease data from 30 countries, enabling interactive exploration of spatial-temporal trends. Users can filter by pathogen, year, and country to visualize clusters, such as the 2022 *Campylobacter* surge in Denmark, which correlated with poultry farm density (R^2 = 0.78).

Predictive modeling for targeted interventions

Machine learning models trained on historical outbreak data and satellite-derived variables (e.g., NDVI for vegetation moisture) achieved 89% accuracy in predicting *E. coli* contamination risks in leafy greens. In Romania, such models guided inspections, reducing outbreak-linked produce by 34% in 2023.

Mitigation strategies by integrating geospatial innovations

EFSA's story maps and dashboards exemplify cross-sectoral data integration, overlaying human case reports with livestock movement permits and water quality indices.

Proposed expansions include Zoonotic Risk Index (a composite metric weighting pathogen prevalence, antimicrobial resistance, and climate vulnerability scores at the NUTS-2 region level) and Automated Syndromic Surveillance (NLP is used to analyze veterinary clinic records and social media for early signals).

Climate-adaptive food safety policies include pathogen-specific early warning systems (deploying sensor networks in high-risk watersheds to monitor *Cryptosporidium* and *Giardia* levels, with real-time alerts to producers) and heatwave-triggered protocols (mandating accelerated testing of RTE foods when regional temperatures exceed 30°C for ≥5 days, mitigating *Listeria* proliferation).

Geospatial capacity building

The WHO European Geospatial Coordination Hub's roadmap prioritizes training programs (a 2024 master's curriculum in spatial epidemiology for public health officials) and common data infrastructures (federated GIS platforms allowing MSs to share anonymized outbreak data without compromising privacy).

Over the past 10 years, there has been an increase in pathogen transmission among human, domesticated animal, and non-domesticated animal populations due to population relocations. This underscores the latent epidemiological risks associated with displacement and highlights the need for targeted prophylactic strategies to mitigate the emergence of zoonotic diseases.

Also, adaptive strategies by animal owners were applied, such as grazing among host communities' livestock or encroaching on wildlife habitat, leading to heightened risks of zoonotic pathogen introduction. These findings emphasize the importance of addressing the welfare and management of livestock in displacement settings to mitigate zoonotic disease transmission risks.

We could also identify the specific zoonotic pathogens prevalent in displaced populations, livestock, and wildlife. These informations would provide insights into the zoonotic disease dynamics in displacement contexts and inform targeted interventions for disease control and prevention.

The lack of primary research addressing zoonoses in displacement contexts is highlighting research gaps and the need for comprehensive studies on zoonotic disease dynamics in displaced populations. This fact underscores the importance of further research to enhance understanding of zoonotic disease risks in displacement settings and improve

public health responses in these vulnerable populations.

The development of a predictive modeling framework that integrates environmental, socio-economic, and displacement-related factors to forecast zoonotic disease outbreaks in displacement contexts are needed.

By utilizing data on displacement patterns, livestock movements, host community interactions, and disease transmission dynamics, this model could help identify high-risk areas for zoonotic disease emergence and inform proactive intervention strategies to mitigate public health threats in displaced populations.

Future efforts should focus on improving reporting mechanisms, enhancing food safety regulations, and fostering collaboration across MSs.

The EFSA and the ECDC provide valuable data on the epidemiology of animal-to-human transmissible diseases within the EU, elucidating key epidemiological trends and areas requiring intervention. While reductions have been observed in specific zoonotic infections, challenges persist, notably concerning *Campylobacter* spp. and emergent *Salmonella* spp. strains.

Interagency cooperation among governing bodies, agricultural producers, distributors, and end-users is essential for establishing food safety.

The EU's foodborne disease landscape has entered a critical phase, where traditional surveillance systems struggle to match the velocity of climate-driven pathogen evolution. Geospatial technologies offer a transformative pathway, enabling predictive analytics, precision interventions, and democratized data access.

However, realizing this potential requires addressing fragmented data standards, legal barriers to cross-border data sharing, and uneven technical capacities across MSs.

Prioritizing investments in geospatial literacy and interoperable platforms will be pivotal in transitioning from reactive outbreak management to preemptive risk mitigation, safeguarding both public health and food security in an era of escalating environmental uncertainty.

CONCLUSIONS

Key findings revealed that population displacements over the past decade have amplified zoonotic pathogen transmission at the human-livestock-wildlife interface, driven by adaptive practices such as shared grazing and habitat encroachment.

Specific pathogens, including *Campylobacter* spp. and emergent *Salmonella* strains, persist as critical threats in displacement-affected regions, while research gaps hinder targeted interventions.

The urgency of these findings lies in the compounding effects of climate change and fragmented surveillance systems, which exacerbate spillover risks and strain public health responses.

Data from EFSA and ECDC underscore persistent zoonotic and foodborne disease burdens in the EU, emphasizing the need for adaptive frameworks to address evolving epidemiological challenges.

Recommendations prioritize developing predictive models that integrate environmental, socio-economic, and displacement data to forecast outbreaks.

Strengthening interagency collaboration, harmonizing food safety regulations, and investing in geospatial technologies (particularly interoperable platforms and data literacy) are critical to transitioning from reactive measures to preemptive risk mitigation.

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PRECISION LIVESTOCK FARMING: MONITORING MICROCLIMATE PARAMETERS IN DAIRY COW SHELTERS

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Abstract

Knowledge of microclimate parameters in dairy cow shelters allows farmers to monitor animal welfare and production process. The use of precision instruments offers facilities: economic, by increasing profit; farmers who can take real-time measures to adjust microclimate parameters. The purpose of this study was to monitor the microclimate parameters in a dairy cattle barn using precision livestock farming tools over a period of 9 months. This study aims to provide information about the environmental conditions in the barn and to identify potential issues to optimize the welfare and productivity of the cows. The data was collected and processed using the BlueMonitor software platform. For each analyzed parameter - temperature, relative humidity, carbon dioxide concentration, particle concentration, and dew point - central tendency statistics (mean) and dispersion statistics (SEM, SD, CV %) were calculated. Differences between monthly averages were tested for significance using the Fisher test, revealing significant variations across all parameters. Monitoring microclimate parameters using precision instruments in animal husbandry enables farmers to take real-time measures, ensuring dairy cows are provided with optimal conditions to express their productive potential.

Key words*: animal welfare, farm animal, microclimate parameters, precision farming.*

INTRODUCTION

The expansion of the global population, alongside mounting strain on natural resources and the impact of global warming, is shaping a new operational environment. Escalating world demand for food, amplified urbanization rates, rising input costs, heightened strain on water resources, and growing vulnerability of crops and animals to climate variations can lead to reduced food production (Mihai et al., 2023). The increasing demand for livestock products has led to challenges related to environmental sustainability, human health, and animal welfare on farms. A critical aspect of ensuring food security revolves around the rearing and utilization of cattle, given that this animal species serves as a primary source of raw material for various food products. Despite a decline in cattle livestock numbers in recent years, milk production has surged. This is attributed to farmers' focus on leveraging animals with high production potential while maintaining optimal conditions for animal welfare (Defta et al., 2023).

The well-being of animals raised for food is heavily influenced by human management practices. Various factors, such as housing and bedding conditions, space availability, transportation methods, stunning techniques, slaughter processes, and practices like castration and tail docking, can significantly affect their welfare. Animal welfare refers to the well-being of animals, encompassing both their physical and psychological health. It involves ensuring that animals are provided with appropriate living conditions, adequate nutrition, proper healthcare, and opportunities to express natural behaviors. Animal welfare also involves protecting animals from physical and mental suffering, distress, and unnecessary harm. Animal welfare is an ethical and moral consideration that emphasizes the responsibility of humans to treat animals with compassion,

respect, and dignity. It is recognized as an important aspect of responsible animal care and is promoted through legislation, regulations, industry standards, and public awareness initiatives (www.efsa.europa.eu).

Dairy cows are often perceived to have better welfare in pasture-based systems, but this cannot always be implemented as a farming system. This highlights the importance of considering different breeding systems and ensuring animal welfare in any farming system (Arnott et al., 2017).

As climate change has become an increasingly frequent topic in global agricultural production, heat stress (Vučković et al., 2019; Bohmanova et al., 2007; West, 2003; ST-Pierre et al., 2003) is a problem in animal husbandry, especially dairy cows (Gauly, 2013; Mihai et al., 2020; Gavrila et al., 2015). In this context, animal welfare (Gavrila et al., 2015) has become an increasingly intense concern for farmers. Monitoring the parameters in the shelter, with the help of Precision Livestock Farming (Chadda et al., 2021), allows obtaining a wide range of information that helps farmers make decisions in real time (Mihai et al., 2020; Lokhorst, 2018; Kelemen et al. 2016) to improve the quality of life of dairy cows and increase production. Also, the precision instruments (Shruthi et al., 2018) in animal husbandry help to know the impact of the dairy cows breeding on the environment (Berckmans, 2014).

Precision Livestock Farming (PLF) technologies have emerged as a promising solution in response to these concurrent needs, namely to ensure the welfare of animals during their intensive breeding. PLF technologies offer farmers opportunities to improve efficiency, reduce environmental impact, and promote animal health and welfare. They provide farmers with valuable insights, real-time monitoring capabilities, and precision management tools to ensure the health, comfort, and overall well-being of livestock. These include health monitoring (animals' health parameters in real-time), behavioral analysis (feeding patterns, movement, and social interactions), environment control (temperature, humidity, and air quality within livestock facilities), nutritional management, and reproductive health (estrus detection and reproductive abnormalities). However, their implementation presents challenges and raises concerns about animal welfare. The legal framework for PLF technologies is important, ensuring that regulations prioritize animal welfare. Initiatives like the Welfare Quality® project in the European Union (EU) promote animal welfare and sustainability (Papakonstantinou et al., 2024; Nica & Vidu, 2023).

While PLF technologies offer opportunities for sustainable livestock production, prioritizing animal welfare is vital for ethical and responsible farming practices.

Consumer demand for ethically produced products emphasizes the need for acknowledging animal dignity and welfare. Consumer education is essential in raising awareness about animal welfare standards and encourage support for products sourced from farms that prioritize animal welfare. It's important to communicate with consumers in a positive manner and respect their decision to pay extra for products from farms where animal welfare is a priority (Nica & Vidu, 2023; Siegrist & Hartmann, 2020; Schillings et al., 2021).

MATERIALS AND METHODS

a. **Data collection instruments**

Considering the specifics of the study, data were collected through the BlueMonitor software platform. The information obtained from the monitoring of the microclimate parameters in the sheds contributed to a good management of the integrated management activities in the Didactic Research and Development Agronomic Center Moara Domnească (dairy farm).

The technical specifications of the sensors are indicated in Table 1.

The calculation formula for determining the dew point:

$$T_d = T - \frac{100 - RH}{5} \quad (1)$$

were:

T_d – the dew point value in degrees Celsius;

T – the air temperature in degrees Celsius;

RH – the relative humidity in percentage.

The BlueMonitor platform offers in a standard Web interface all the design and working tools for SCADA automation applications (www.bluemonitor.ro). The BlueMonitor SCADA application uses PostgreSQL9.X as a database, for which customized settings can be applied considering the volume of processed data. The settings allow the response time improvement and, consequently, the speed of displaying data in the interface.

Table 1.Technical specifications of sensors

System specifications			
Model CL-200 (**temperature, CO_2, RH, PM2.5**)			
Real time clock	Yes		
Interface	RS-485		
Dimension (DxH)	Ø 150 mm x 53 mm		
Enviroment	Operating temperature	0 to +50°C	
	Storage temperature	-30 to +75°C	
	Humidity	10 to 90% RH Non-condensing	
Model ME3-NH_3 (**ammonia**)			
Response time (T_{90})	≤ 60 s		
Interface	RS485, MODBUS-RTU		
Enviroment	Operating temperature	-20 to +50°C	
	Storage humidity	15% to ~90% RH	
I/O specifications			
Sensor category	**Range**	**Accuracy**	**Resolution**
Temperature	-10 to +50°C	± 0.6°C	0.1°C
Relative humidity	0 to 100%	± 5%	0.1%
Carbon dioxide	0 to 9999 ppm	± 40 ppm ± 3% of measured value	1 ppm
Particle concentration	0~400 µg/m^3	-	1 µg/m^3
Ammonia	0-100 ppm	-	0.5 ppm
Dew point	Calculated using temperature and relative humidity		0.1°C

b. **Study area and material**

The study was carried out in the Didactic Research and Development Agronomic Center Moara Domnească (dairy farm), where Montbéliarde dairy cows are raised and managed through husbandry practices (25 dairy cows with an average body weight of 673 kg and an average daily milk production of 15 l per cow).

Information about the shelter:

- Dimensions: L = 79.5 m; l = 9.6 m; height up to the roof = 2.65 m; height of the roof = 5.5 m;
- Manure management: manure is collected from the barn using a scraper blade and temporarily stored in the septic pit;
- Ventilation system: the barn is equipped with a natural ventilation system. Each side wall has 24 ventilation windows (80 cm/110 cm) that allow air intake. The roof is equipped with 9 air exhaust openings (9 ventilation chimneys). Both front walls have 2 openings (80 cm/110 cm) at the top (in the roof area).

Microclimate and ventilation are important factors by which the air quality in dairy cow shelters is identified (Herbut et al., 2018). Good ventilation ensures a low concentration of harmful gases (Armstrong, 1994; Parois et al., 2018; Zou et al., 2019) and thermal comfort for dairy cows (Teye, 2008; Teye & Hautala, 2008).

In the shed of adult cows (free stall), sensors that monitor the air quality in the shelter are installed: temperature, relative humidity, ammonia, carbon dioxide and particle concentration.

c. **The investigated parameters:** temperature, relative humidity, ammonia, carbon dioxide, particle concentration and dew point: period in which data were collected: April-December.

d. **Data statistical analysis**

The values of the microclimate parameters in the shed were recorded through the BlueMonitor platform. Descriptive and

inferential statistics were used for data processing through:

- *Techniques of data organization and summative presentation*: numerical techniques: simple frequency analysis; graph;
- *Summative numerical indicators* of central tendency (mean) and dispersion (standard error of the mean- SEM, standard deviation - SD and coefficient of variation - CV %).

To test the significance of the differences in average amounts (Fisher, $p< 0.05$), 6 statistical hypotheses were formulated:

H1: Variations in temperature lead to differences between monthly averages;
H2: Variations in relative humidity lead to differences between monthly averages;
H3: Variations in ammonia concentration lead to differences between monthly averages;
H4: Variations in carbon dioxide concentration lead to differences between monthly averages;
H5: Variations in particle concentration lead to differences between monthly averages;
H6: Dew point variations lead to differences between monthly averages.

RESULTS AND DISCUSSIONS

BlueMonitor is an online *software* platform developed by *BlueNote Communications*, which offers a constantly updated image of monitored microclimate factors and implements procedures and applications for specific collected data processing (e.g. graphics).

The BlueMonitor platform offers in a standard Web interface all the design and exploitation tools for SCADA automation applications (www.bluemonitor.ro): web interface, through a standard *browser*; multi-role system of usage rights; interactive graphic objects; flexible alerts; automatic calculation of derived values (there are a number of functions with the help of which simple operations can be performed for the data collected through the sensors); custom charts and reports; detailed logs (helps to record system events and user activity in the interface) (Figure 1).

For each of the analyzed parameters, the specialized studies refer to maximum allowed limits (temperature: 10-14°C, humidity: 60-75%; carbon dioxide: 3000 ppm; ammonia: 20 ppm; particle concentration 500 particles/cm^3), so that the animals can have a healthy and productive life. The results obtained after data processing were compared with the standard values and those from the specialized literature (Vučković, 2019; Ngwabie et al., 2009; Strmeňová et al., 2015).

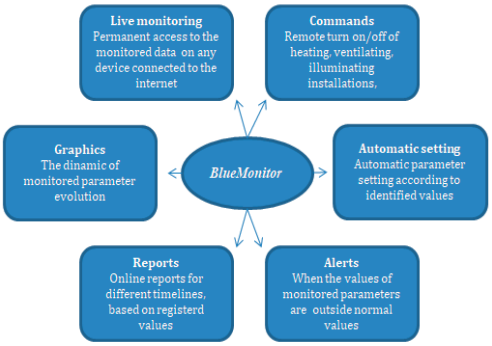


Figure 1. BlueMonitor monitoring facilities

Temperature

Cows are able to adapt to variable temperature and humidity conditions throughout the year (Kadzere et al., 2002). West (2003) states that this aspect can be confirmed through a fairly wide range both for temperature (-0.5°C to 20°C) and for humidity (60-80%). These ranges represent a thermoneutral zone that does not significantly induce physiological or behavioral changes in dairy cows. The critical level of shelter air temperature is generally considered to be in the range of 25-26°C (West, 2003) or 24-27°C (Brouček et al., 2009). The welfare of dairy cows is affected by higher values.

The temperature inside the shed is influenced by the time of year. For each month two days have been identified as monthly temperature maximum and minimum value.

Temperature monitoring has allowed the calculation of corresponding average values for each day and on a monthly level. For April, the temperature varied between 16.43-18.81°C (daily average), with an average of 16.95°C (monthly average). For this month the temperature values sent by the sensors did not exceed the permitted limits for this parameter (Figure 2).

In May, the highest temperature was generated by the system for May 24th, namely 24.05°C (daily average), with approx. 9°C more

compared to the standard maximum allowed for thermal comfort in the dairy cow shed.
From the data stored in the system, an average temperature for June of 23.85°C was determined, with limits between 17.77°C (daily average) and 29.39°C (daily average).

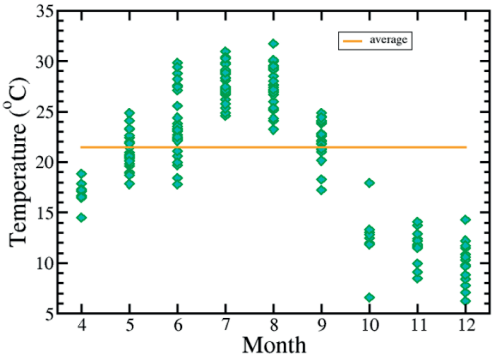


Figure 2. Temperature - daily average values corresponding to each month

July and August were the hottest months of the year. The average temperature in the shelter for these months was approx. 28°C, with a maximum of 31.69°C (daily average). For these months, the allowed limits have been exceeded. Notifications/alerts were received from the BlueNote platform, which enabled timely optimal measures to prevent hot air from entering the litter inside the shelter. The fact that measures were taken to recover the temperature inside the shelter also helped to reduce heat stress. The high temperature during this period was reflected in productivity.
For September, based on the data taken from the sensors in the shelter, a daily average temperature of approx. 22.18°C was registered, the maximum recorded for this month being 24.85°C (daily average), and the minimum 17.2°C (daily average).
As the warm season passed, an optimal temperature in the shelter was easier to maintain (Table 2).
The average temperature determined for the month of December was 9.75°C. The sensors in the shelter registered values that fall within the allowed limits for thermal comfort, i.e. the temperature varied between 6.22°C (daily average) and 14.28°C (daily average).
Through the analysis of variance by comparing the monthly means for the temperature, there were detected statistically significant differences ($p < 0.05$).

Table 2. Temperature: statistics and Fisher test

Month	Descriptive statistics		
	MEAN ± SEM	SD	CV %
April	16.95 ± 0.32	1.06	6.24
May	21.08 ± 0.30	1.68	7.98
June	23.85 ± 0.59	3.21	13.48
July	27.82 ± 0.29	1.60	5.76
August	27.30 ± 0.38	2.15	7.86
September	22.18 ± 0.41	1.94	8.76
Octomber	12.45 ± 1.09	3.09	24.79
November	11.60 ± 0.42	1.57	13.55
December	9.75 ± 0.50	2.08	21.31
Significance between months		191.2*	

Note: Fisher test, $p < 0.05$

Relative humidity

Monitoring the relative humidity is important especially in the warm season. The decrease in humidity in combination with an increased temperature influences the increase in the concentration of harmful gases.
Investigating the data stored in the BlueNote system, a high degree of variability was observed throughout the months investigated (Figure 3). The average of the investigated months was 77.43%.

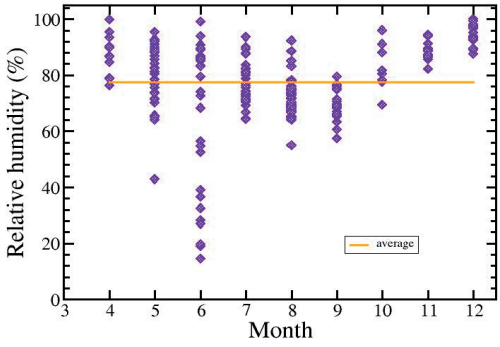


Figure 3. Relative humidity - daily average values corresponding to each month

For each month of the year, the average was determined, which varied between 66.63% (June) and 94.49% (December). It was observed that only in April, October, November and December the maximum value accepted as optimal for the microclimate in the shelter was exceeded.
In June, the lowest relative humidity was identified, namely lower by 12.74% compared

to the previous month and 10.35% compared to the following one. For the values in June, a high degree of variability was observed, three days (daily average) with a very low humidity (approx. 17.75%) compared to the allowed limit were identified. The coefficient of variation for this month was very high (40.44%), indicating high variability for this month's group of statistical units. The average determined for this distribution is not representative, as the coefficient of variability is higher than 30%. Analyzing each daily average for this month, humidity ranges from 14.57% to 99.13%.

The most homogeneous distribution of values was for the month of December, with a 3.93% coefficient of variability. It can be considered that the average determined for each of the value distribution, corresponding to the investigated months (except June) is representative for the statistical population, the value of the coefficient of variability being in each case less than 15%.

After a comparative analysis of the values for standard error of the mean (SEM) from the nine distributions corresponding to the investigated months, it was noticed that in June the standard error of the mean is approximately 3 times higher compared to the months of July, August, September and November and double compared to April and May. The most homogeneous distribution was determined for the month of December, with the standard error of the mean of 0.90 (Table 3).

Through the analysis of variance by comparing the monthly averages for the relative humidity, there were detected statistically significant differences ($p<0.05$).

Table 3. Relative humidity: statistics and Fisher test

Month	Descriptive statistics		
	MEAN ± SEM	SD	CV %
April	87.32 ± 2.23	7.39	8.46
May	79.37 ± 2.05	11.39	14.35
June	66.63 ± 4.92	26.95	40.44
July	76.98 ± 1.37	7.63	9.90
August	72.66 ± 1.34	7.48	10.29
September	69.16 ± 1.16	5.45	7.89
Octomber	82.81 ± 3.01	8.51	10.27
November	89.26 ± 1.01	3.78	4.24
December	94.49 ± 0.90	3.71	3.93
Significance between months 11.297*			

Note: Fisher test, $p < 0.05$

Ammonia

The ammonia concentration of the air in shelters is influenced by several factors, including: the amount and composition of manure and the period of stagnation in the shelter, the pH of the manure, the density of animals in the shelter etc.

In 2015, Strmeňová measured the ammonia concentration in two areas of a dairy cow shelter. In front of the feeding area, the average concentration was recorded as 10.89 ppm, while in the middle area between two rows of berths, the average concentration was 3.6 ppm.

The maximum value of the ammonia concentration recorded by the sensors in the shelter during the analyzed months was 18.2 ppm (daily average), in December (Figure 4).

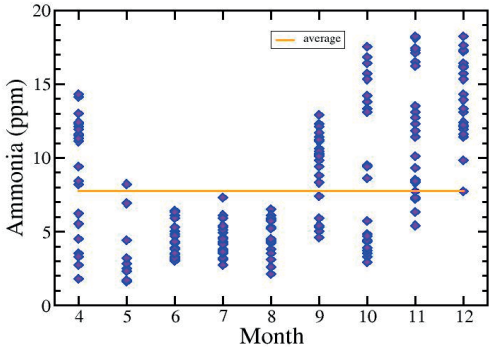


Figure 4. Ammonia concentration - daily average values corresponding to each month

The dynamics of ammonia concentration was investigated over nine months. The average for each month was determined and it was observed that the highest concentration was in December, respectively 11.15 ppm more compared to the month of May for which the lowest average was calculated. For June and July, the ammonia concentration averages were similar, with a variation between 4.33 ppm - 4.62 ppm.

The investigation of data from the BlueNote platform allowed for the establishment of an average for the investigated months (7.74 ppm). Compared to the average determined for the 9 months investigated, for the monthly averages, maximum variations of 6.44 ppm (December) and minus 4.71 ppm (May) were determined.

Fluctuations of the ammonia content in the air reflect in the degree of heterogeneity of the characteristic data groups of each month. The highest degree of variability was observed in October, the standard error of the mean for this month having the highest value (5.34). This aspect is also marked by the high value of the coefficient of variability (60.36 %). Comparing the results obtained for the coefficient of variability determined for each of the nine investigated months, it was observed that in December were the smallest fluctuations in the concentration of ammonia (Table 4).

Through the analysis of variance by comparing the monthly means for the ammonia, there were detected statistically significant ($p < 0.05$).

Tabel 4. Ammonia statistics and Fisher test

Month	Descriptive statistics		
	MEAN ± SEM	SD	CV %
April	8.63 ± 0.86	4.21	48.79
May	3.03 ± 0.30	1.51	49.60
June	4.62 ± 0.25	1.24	26.84
July	4.33 ± 0.23	1.11	25.78
August	4.45 ± 0.22	1.10	24.69
September	9.40 ± 0.54	2.59	27.60
October	8.85 ± 7.09	5.34	60.36
November	12.09 ± 0.85	4.21	34.82
December	14.18 ± 0.57	2.79	19.73
Significance between months		37.60*	

Note: Fisher test, $p < 0.05$

Carbon dioxide

The CO_2 concentration in the shelter varies depending on the amount of manure and litter in the shelter, the number of animals/m^2 and of course the quality of the atmospheric air.

In 2015, Strmeňová measured the CO_2 concentration in two areas of a dairy cow shelter. In front of the feeding area, the average concentration was recorded as 2121.50 ppm, while in the middle area between two rows of berths, the average concentration was 1856.50 ppm.

Accessing the BlueNote interface allowed the identification of a maximum value during the investigated months of 1242.43 ppm (the daily average for May^{1st}) (Figure 5).

For each of the investigated months, an average was determined based on the stored data, and the value distribution curve has a concave shape. The highest averages for the carbon dioxide content of the shelter air were determined in April and December.

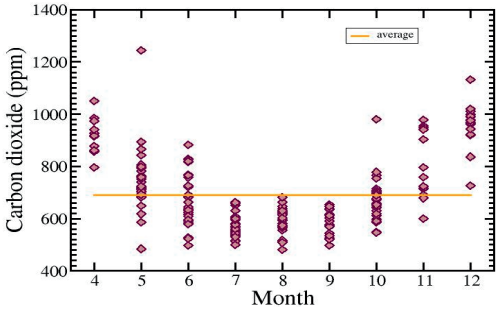


Figure 5. Carbon dioxide concentration - daily average values corresponding to each month

The largest fluctuations in the content of CO_2 in the air were monitored for May, with variations between 483.27 ppm (daily average) and 1242.43 ppm (daily average). This aspect is also confirmed by the high value of the variability coefficient (16.72%) and the standard deviation (125.89), which varies directly proportional to the degree of variability. The situation was similar in November.

The most homogeneous value distributions were recorded in April and August, with the coefficient of variability having a value below 8%. Similar situations were also determined in July, September, and December, however, for these months the variability coefficient was between 8.06-8.85% (Table 5).

Table 5. Carbon dioxide: statistics and Fisher test

Month	Descriptive statistics		
	MEAN ± SEM	SD	CV%
April	916.88 ± 21,13	70.09	7.64
May	752.54 ± 22,61	125.89	16.72
June	673.54 ± 18,11	99.23	14.73
July	568.67 ± 8,39	46.72	8.21
August	596.45 ± 7,84	46.76	7.84
September	584.30 ± 10,05	47.14	8.06
October	667.59 ± 14.64	81.54	12.21
November	793.78 ± 33,33	124.73	15.71
December	955.68 ± 20,52	84.63	8.85
Significance between months		59.08 *	

Note: Fisher test, $p < 0.05$

The lowest CO_2 concentrations were recorded in July, August, and September. The most likely explanation is that during those months, the cows spent more time in the outer paddock (Table 5).

After a comparative analysis of the monthly average values, it was observed that CO_2 varied

between 568.67 ppm (monthly average - July) and 955.68 ppm (monthly average - December), with a maximum difference from the investigated months average of 260.74 ppm (Table 5).
Through the analysis of variance by comparing the monthly means for the carbon dioxide, there were detected statistically significant differences ($p<0.05$).

Particle concentration

Particle concentration is an indicator of indoor air quality. According to the data recorded by the sensors in the shelter, the daily average on April 25rd, the maximum concentration in particles was 122.01 µg/m^3 (Figure 6).

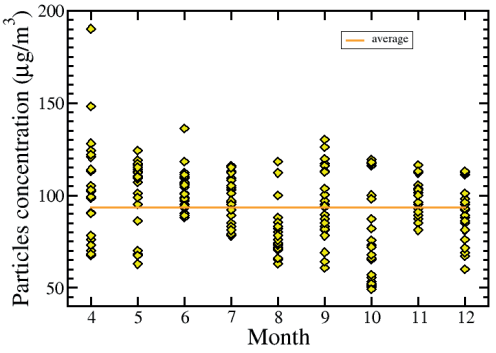


Figure 6. Particle concentration - daily average values corresponding to each month

Monthly averages vary between 74.27 µg/m^3 (October) and 103.06 µg/m^3 (April). The average of the analyzed months was 93.41 µg/m^3, with a maximum difference of 19.14 µg/m^3 compared to the monthly averages. A comparative analysis of the monthly averages, shows that higher values were observed in April (9.65 µg/m^3), May (7.86 µg/m^3), June (8.53 µg/m^3), July (6.25 µg/m^3) and November (5.41 µg/m^3), respectively lower in August (14.41 µg/m^3), September (0.97 µg/m^3), October (19.14 µg/m^3) and December (3.19 µg/m^3) compared to the average of the analyzed months (Table 6).
The comparative analysis of the values shows that the standard error of the mean (SEM) varies within very small limits, namely between 2.16 and 5.87, which indicates the homogeneity of the values recorded by the sensors in the shelter.

During summer months, the difference between the averages recorded for the particle concentration was of maximum 22.94 µg/m^3.

Table 6. Particle concentration: statistics and Fisher test

Month	Descriptive statistics		
	MEAN ± SEM	SD	CV %
April	103.06 ± 5.87	28.76	27.91
May	101.27 ± 3.76	18.44	18.21
June	101.94 ± 2.20	10.79	10.59
July	99.66 ± 2,52	12,36	12.40
August	79.00 ± 2.83	13.90	17.60
September	92.44 ± 5.42	26.57	28.74
Octomber	74.27 ± 5.01	24.57	33.08
November	98.82 ± 2.16	10.62	10.75
December	90.22 ± 3.01	14.76	16.36
Significance between months	7.271*		

Note: Fisher test, $p < 0.05$

Through the analysis of variance by comparing the monthly means for the particle concentration, there were detected statistically significant differences ($p<0.05$).

Dew point

Knowing the dew point, temperature and humidity values allows the temperature humidity index (THI) to be determined. Variations of this index influence the installation of thermal stress.
The investigation of the database collected (for temperature and relative humidity) by the sensors installed in the dairy cows shed at the Moara Domnească farm revealed a maximum monthly average of 23.22°C, in July. Average monthly values range between 8.65°C (December) and 23.22°C (July). A daily average for the dew point was established based on data recorded by sensors for temperature and relative humidity (Figure 7).

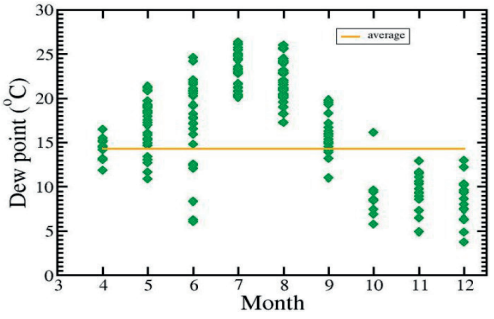


Figure 7. Dew point - daily average values corresponding to each month

The average of the analyzed months for this microclimate parameter was 16.92°C. As expected, the lowest values were recorded in the cold months of December, with a maximum difference of 8.27°C from the average of the investigated months (Table 7).

Table 7. Dew point: statistics and Fisher test

Month	Descriptive statistics		
	MEAN ± SEM	SD	CV %
April	14.42 ±0.39	1.31	9.09
May	16.95 ± 0.52	2.90	17.12
June	17.17 ± 0.92	5.04	29.35
July	23.22 ± 0.35	1.97	8.51
August	21.84 ± 0.40	2.24	10.30
September	16.01 ± 0.48	2.29	14.31
October	9.01 ± 1.11	3.15	35.02
November	9.45 ± 0.57	2.16	22.93
December	8.65 ± 0.59	2.45	28.36
Significance between months		64.958*	

Note: Fisher test, $p < 0.05$

Through the analysis of variance by comparing the monthly means for the dew point, there were detected statistically significant differences ($p<0.05$).

Interrelationships between microclimate parameters

a. ***Temperature, humidity and dew point***

Microclimate factors are closely interdependent, influencing animal well-being and implicitly productivity. The comparative analysis of the values for temperature and dew point shows that as the temperature increases, so does the dew point, i.e. a directly proportional variation. In contrast, an inversely proportional variation was observed between relative humidity and dew point.

The most homogeneous value group, both for relative humidity and dew point, was for the 30-35°C temperature range. This aspect is indicated by the very small value of the coefficient of variation (4.26% for relative humidity and 2.45% for dew point) (Table 8).

The highest coefficient of variation (33.03%) for the dew point parameter was determined for the temperature range 20-<25°C, which indicates a high degree of variation between the values (temperature and relative humidity) recorded by the sensors in the shelter for this temperature range (Table 8).

The value obtained for the coefficient of variation corresponding to the temperature range 6-<10°C and 25-<30°C, being higher than 15% but lower than 30% indicates that the average determined for this microclimate parameter has a moderate representativeness for the respective value group (Table 8).

b. ***Temperature and ammonia***

Temperature can be treated as a key factor for an optimal microclimate due to its impact on ammonia emissions (Mulvaney et al., 2008) compared to the impact of manure removal frequency, floor condition, cleanliness and feeding activity of cows (Zhang et al., 2005). Emission rates are influenced by the type of flooring and manure handling method (Parois et al., 2018). The lowest ammonia emission was determined for shelters with solid floors, smooth surfaces, draining, and exhaust with scraper or plow scraper (Zhang et al., 2005). In the shelter where microclimate parameters were monitored, the floor is made of concrete, the resting areas are equipped with rubber mats, and manure is removed with a scraper blade.

These features are part of the factors ensuring a low concentration of ammonia. Comparing values based on the months analyzed for temperature and ammonia, it was observed that an increase in temperature does not always lead to an increase in ammonia concentration.

Table 8. Relative humidity and dew point correlated with temperature

Temperature	Descriptive statistics					
	MEAN ± SEM		SD		CV %	
	Relative humidity	Dew point	Relative humidity	Dew point	Relative humidity	Dew point
6-<10°C	91.81 ± 1.46	7.03±0.42	5.29	1.51	5.76	21.61
10-<15°C	89.51±1.41	10.54±0.31	7.21	1.60	8.06	15.21
15-<20°C	83.86±2.31	15.22±0.52	10.86	2.45	12.95	16.11
20-<25°C	78.31±1.24	19.78±0.84	9,59	6.53	12.25	33.03
25-<30°C	74.92±1.11	23.56±0.69	7.83	4.81	10.46	20.42
30-35°C	74.62±1.42	25.55±0.28	3.18	0.63	4.26	2.45

For example, during the warmest months analyzed (July and August), the cows spent a lot of time in the paddock, which resulted in a reduction in manure volume and low ammonia concentration. In December, which had the lowest monthly average temperature, the monthly average ammonia concentration was highest due to reduced ventilation from closing the windows.

To reduce ammonia emissions from dairy cow shelters, it is important to manage air circulation and optimize temperature, especially during high temperature periods (Ngwabie et al., 2009). Temperature control is necessary in order to not create the possibility of hot air entering the litter inside the stable. Decreasing the average annual temperature by 1°C would reduce the annual ammonia emission by 5% (Bleizgys et al., 2013). Lowering the temperature in dairy cow sheds will lead to lower pH levels and biological processes that create ammonia in manure (Teye & Hautala, 2008). Seasonal temperature fluctuation and the initial pH of the floor surface play an important role in the amount of volatilized ammonia (Mulvaney et al., 2008).

The specific risk occurs when the temperature exceeds 20°C. An increase of temperature contributes to the intensification of ammonia emissions (Bleizgys et al., 2013). The variation in ammonia concentration is closely related to the variation in carbon dioxide concentration. There are studies that demonstrate that there is a positive correlation between these two gases (Ngwabie et al., 2009; Strmeňová et al., 2015).

c. ***Relative humidity and the concentration of harmful gases***

Relative humidity influences the concentration of harmful gases. For example, there are studies that concluded that when the temperature increased and the humidity decreased, the concentration of harmful gases increased (Strmeňová et al., 2015).

d. ***Consequences of the interaction of factors***

Another aspect frequently analyzed in dairy cow studies is related to heat stress. Heat stress is defined as the sum of external factors acting on an animal, leading to an increase in body temperature and causing a physiological response/reaction (Dikmen & Hansen, 2009). The problem of heat stress is associated with high air temperatures combined with high relative humidity in cow shelters (Hill & Wall, 2015).

Under unfavourable thermal conditions, dairy cows can dissipate body heat mainly by increasing their respiration rate and reducing the feed intake and milk production. In these situations, dairy cows try to maintain their homeostasis by seeking shade, reducing feed consumption and movements (Schutz et al., 2009).

It is essential to create conditions that allow the dissipation of dairy cows' body heat, as hyperthermia has a negative impact on the cow's health and therefore on productivity and reproductive activity. When the upper critical temperature is exceeded, the adaptation mechanisms fail to remove the generated excess heat.

Studies conducted in shelters and on pastures have shown that among the environmental conditions with the highest influence on the welfare of dairy cows are temperature and relative humidity, and that these can be ensured by applying appropriate solutions (Janni & Allen, 2001).

CONCLUSIONS

The average temperature was 21.43°C, with significant monthly fluctuations. Alerts were sent when temperatures exceeded comfort limits, allowing quick corrective actions. Relative humidity averaged 77.43%, with a 27.86% variation, requiring management to prevent humidity-related issues. Ammonia levels peaked in December and were lowest in May, averaging 7.74 ppm, with significant monthly differences ($p<0.05$). CO_2 concentrations also varied significantly ($p<0.05$), highest in December (955.68 ppm) and lowest in July (568.67 ppm), influenced by seasonal cow behavior. Particle concentration peaked in April and was lowest in October, averaging 93.41 $\mu g/m^3$. The average dew point was 16.92°C, lowest in December. Monitoring these parameters is essential for preventing thermal stress and ensuring dairy cow welfare.

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APPLICATION OF MEDICINAL PLANTS, PROBIOTICS AND SYNBIOTIC PRODUCTS IN PREVENTIVE CARE AND ALTERNATIVE THERAPY FOR FARM ANIMALS IN MODERN VETERINARY MEDICINE. A REVIEW

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Abstract

Sustainable production of high-quality animal and plant production for feeding the growing world population of consumers is a key challenge for the future industry. New animal farming methods are introduced, focusing on enhancing meat and milk quality and safety, in parallel taking into account welfare and preservation of natural environment. Selection conducted towards high growth rate results in breeds and hybrids characterized by fast growth but in the same time by delayed morphological and functional maturity and by an underdeveloped immune system. Antibiotics and other medicinal products are widely used, mainly to modify the digestive microbiota, to increase the productivity and growth of animals and to compensate for low immunological activity. The long-term use of these substances has led to the development of drug-resistant microorganisms, posing a threat to consumer health and having a negative impact on the environment. This review studies the ways of limiting the use of antibiotics in animal husbandry, alternative strategies to improve production and animal health - the use of probiotic, prebiotic and symbiotic preparations, as an effective treatment against pathogens.

Key words*: antibiotic resistance, farm animals, medicinal plants, prebiotics.*

INTRODUCTION

Antimicrobial resistance (AMR) poses a significant risk to global health and development. One of the primary factors contributing to the rise of resistance in pathogens is the excessive use of antimicrobials. Synthetic drugs have residual effects in animal production (Dowarah, 2017). Reducing their use in animal husbandry is important to maintain antibiotic effectiveness in the future (Lalouckova et al., 2021). The World Health Association has identified AMR as one of the ten major threats to public health, based on the fact that an estimated 1.27 million deaths worldwide in 2019 can be attributed to it (Murray et al., 2022). In 2021, The European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) have published the third joint report on an integrated analysis of antimicrobial consumption and the emergence of antimicrobial resistance. The links between antimicrobial use (AMU) and the development of AMR in commensal *E. coli* in animals are analysed (ECDC, EFSA & EMA, 2021). In Canada, a study based on data from studies in broiler chickens, pigs and turkeys found associations between increasing AMU and decreasing susceptibility of *E. coli* to several antimicrobials (Agunos et al., 2021). Their use was found to be associated with resistant *E. coli* isolates in pig farms in the United Kingdom (Abuoun et al., 2020). Fonseca et al. (2023) examined *E. coli* in calves and found that those treated with sulfonamides, tetracycline, and fluoroquinolones exhibited a higher prevalence of antimicrobial resistance (AMR) and an increased expression of virulence genes in these isolates compared to untreated animals. Similarly, Formenti et al. (2021) reported a greater occurrence of resistant *E. coli* in calves that received treatment. Furthermore, Pires et al. (2022) analyzed public databases from 1980 to 2018, which indicated a notable rise in AMR in commensal *E. coli* in animals, with significant increases for various antimicrobials, notably penicillin combined with β-lactamase inhibitors and cephalosporins, alongside a doubling of multidrug resistance (MDR). In a different investigation in Quebec, Canada, researchers assessed AMR in *E. coli* from fecal samples of

healthy dairy cattle and discovered elevated resistance levels to tetracycline, sulfisoxazole, and streptomycin (Massé et al., 2021). The incidence of antimicrobial resistance in *Salmonella* spp. has been found to be increasing over the past few decades and has caused concern in both human and veterinary medicine (Besser et al., 2000; Fey et al., 2000).
This review examines ways to limit the use of antibiotics in livestock production, alternative strategies to improve production and animal health - the use of probiotic, prebiotic and symbiotic preparations as effective treatments against pathogens.

MATERIALS AND METHODS

In the present review was used information from publications obtained on the Internet - Google Scholar but mostly Research Gate, PubMed, Scopus and Web of Science - as well as journal editions worldwide and also editions in Bulgaria.

RESULTS AND DISCUSSIONS

The use of antibiotics in farming practices is adding to the growing challenge of antimicrobial resistance worldwide (Chang et al., 2015). Animals are treated with significant amounts of drugs, making them reservoirs of bacterial antimicrobial resistance genes. They can transmit to humans via the food chain, through direct contact, and from the environment (Laxminarayan et al., 2013; Lalouckova et al., 2021). This necessitates the search for alternative strategies to improve animal production and health, and in recent years, research has been conducted using probiotic, prebiotic and synbiotic preparations as an effective model in the fight against pathogens. They influence a number of metabolic processes, support the body's immunological response and suppress inflammatory processes. The primary motivation behind their application is the aim to obtain positive outcomes akin to those produced by antibiotic treatments (Markowiak & Śliżewska, 2018).
The term “probiotic” was first introduced in an article by Vergin in 1954. Lilly & Stillwell (1965) defined probiotics as microorganisms that promote the growth of other microorganisms. Fuller (1989) added that for substances to be considered probiotics, they must be viable and exert beneficial effects on the host. Guarner & Schaafsma (1998) further clarified that the dosage of these probiotic organisms is crucial to achieving a positive outcome. Their main function is that they have an effect on the development of the microbiota and ensure a balance between pathogens and bacteria. In *in vitro* studies, it has been demonstrated that low molecular weight compounds, such as hydroperoxides and short-chain fatty acids produced by probiotic microorganisms, play a role in inhibiting the growth of harmful pathogens (Oelschlaeger, 2010). Probiotic products may contain one or more microbial strains, such as Gram-positive bacteria: *Bacillus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, some species of fungi and yeasts of *Saccharomyces cerevisiae* and *Kluyveromyces*, spores of the genus *Bacillus* and strains *Lactobacillus farciminis* and *Pediococcus acidilactici* (Simon, 2005). A number of scientists have established an effect on feed intake, body weight gain and general health in different animal species (Torres-Rodriquez et al., 2007; Samli et al., 2007; Casey et al., 2007; Chiofalo et al., 2004; de Rezende et al., 2012). Numerous scientific publications conclude that their addition to feed leads to improved quantity and quality of milk, meat and eggs and reduces the effect of weak limbs in broiler chickens (Musa et al., 2009; Plavnik & Scott, 1980). The lack of side effects is a significant benefit of probiotics (Markowiak & Śliżewska, 2018).
Weaning is a stressful time for piglets in pig farming, the feeding pattern changes and this leads to a disruption of immunological functions and a negative effect on the balance of the intestinal microbiota (Modesto et al., 2009). In their study, Böhmer et al. (2006) added the probiotic strain. From the 90th day of pregnancy until the 28th day of lactation, the addition of *Enterococcus faecium* DSM 7134 to the sow's feed significantly enhanced feed intake, increased the size of the offspring, and boosted the weight of the experimental animals. Kyriakis et al. (1999) found a positive effect when studying the effect of a probiotic with *Bacillus licheniformis* spores in piglets

from 5 to 10 days old. The study of the literature data shows that probiotics can be successfully used to prevent diseases such as salmonellosis, campylobacteriosis or coccidiosis.

Mountzouris et al. (2007) conducted research on the impact of probiotics in broiler chickens. Their study featured a supplement that included two strains from the *Lactobacillus* genus, along with one strain each of *Bifidobacterium*, *Enterococcus*, and *Pediococcus*. The result of the application of the probiotic leads to the stimulation of the growth of the birds and modulates the composition of the intestinal microbiota (Mountzouris et al., 2007). Recent research has highlighted the benefits of using feed enriched with the probiotic YEA-SACC-1026, which includes the bacterial strains *Bacillus licheniformis* and *Bacillus subtilis*, for sheep. This supplementation was shown to positively impact the fat and protein content of their milk, while also contributing to increased body weight in lambs (Kritas et al., 2006). Additionally, a study by Yu et al. (1997) explored the effects of incorporating *Aspergillus oryzae* into cattle diets, revealing enhancements in milk productivity, specifically in terms of higher protein levels and an increase in dry non-fat solids in the milk. A number of studies indicate that these feed additives contribute to increasing egg production and quality (Haddadin et al., 1996; Kurtoglu et al., 2004) and to reducing their contamination with *Salmonella* (Van Immerseel et al., 2006). In the studies of Haddadin et al. (1996), birds were fed feed supplemented with *Lactobacillus acidophilus*, higher feed conversion rates and increased egg production were found. In addition to probiotics, in recent years the attention of scientists has been attracted by natural products of plant origin - prebiotics or so-called medicinal plants. Prebiotics are types of oligosaccharides that our bodies can't digest but are crucial for the health of our gut microbiota. They play a key role in promoting the growth and activity of beneficial bacteria in the intestines while also positively influencing overall health (Rioux et al., 2005). Research shows that prebiotics can reduce the presence of harmful bacteria in both animal studies and clinical trials involving humans. They eliminate the need for competition with bacteria (Sivieri et al., 2014). In recent years, their use in animal nutrition has received increasing attention, with the effect on overall health being studied (Markowiak & Śliżewska, 2017). Medicinal plants can provide host resistance to colonization by pathogenic bacteria by inhibiting their adhesion to the intestinal epithelium. Indigestible oligosaccharides have been shown to limit intestinal colonization by *Clostridium difficile in vitro* (Hopkins & Macfarlane, 2003). The makeup of gut microbiota is intricately linked to the overall health of an individual. Changes in this composition can lead to local dysbiosis and a range of metabolic disorders. This underscores the importance of investigating new protective and therapeutic approaches to prevent intestinal dysbiosis (Allaw et al., 2020; Lalouckova et al., 2021).

Smiricky-Tjardes et al. (2003) investigated the effect of a prebiotic on the gastrointestinal microbiota in pigs and found a significant increase in the number of *Bifidobacterium* and *Lactobacillus* in feces.

Scientific studies by Shivanna et al. (2013) indicate that in rats fed stevia, a decrease in blood glucose, ALT and AST was found, which is also confirmed by a study by Nenova & Enchev (2022) in pigs. It reduces the concentration of MDA (malondialdehyde) in the liver and improves its antioxidant status. A study by Pirgozliev et al. (2021a) proves that the addition of stevia improves the hepatic antioxidant status in broilers, by increasing hepatic vitamin E and carotenoidite, respectively. Other scientific experiments have found that adding stevia and sea buckthorn to poultry feed can improve their antioxidant status (Pirgozliev et al., 2021b; Pirgozliev et al., 2023). The antibacterial effect of stevia extracts against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* was investigated by Pól et al. (2007) and Lalouckova et al. (2021). Nilson et al. (2023) investigated the effect of stevia extract as a substitute for antimicrobial drugs in broiler chickens. Their studies found improved immunological maturity in primary lymphoid organs and a balanced gut microbiota.

Gęgotek et al. (2018) found that when fibroblasts were incubated with sea buckthorn

oil, there was a notable decrease in the production of reactive oxygen species (ROS). Sea buckthorn supplementation can be used as a natural source of antioxidants for the prevention and treatment of diseases associated with oxidative stress (Zhen et al., 2022). In vivo experiments in mice showed that it has antihyperlipidemic effects. A study by Zhen et al. (2022) showed that a 70% methanol extract effectively reduced leg swelling and inflammation in rats. Similarly, Verma et al. (2011) reported that an extract from sea buckthorn leaves was remarkably effective against 67 different Gram-positive bacteria, managing to inhibit the growth of strains like *S. aureus*, *S. epidermidis*, *S. intermedius*, and *S. pyogenes* by nearly 50%. Carotenoids such as β-carotene, lutein, lycopene, and β-cryptoxanthin exhibit hepatoprotective activity by reducing oxidative stress and regulating lipid metabolism in hepatocytes (Elvira-Torales et al., 2019).

Derbakova et al. (2016) investigated the effect of sea buckthorn extract on *Cryptosporidium* spp. in calves. They reported that it has anti-inflammatory, anthelmintic and antibacterial effects. Its addition to the feed ration leads to improved body weight in pigs, milk production in goats and egg laying in poultry (Shaker et al., 2018).

Recent studies indicate that various plant extracts can enhance the antibacterial effects of conventional medications when tackling mastitis. In their research, Srichok et al. (2022) explored the antibacterial properties of *O. tenuiflorum* extract and its potential interactions with standard antibacterial treatments aimed at mastitis-causing pathogens such as *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus agalactiae*, and *Escherichia coli*. Antibacterial drugs in combination with plant extracts broadened the antibacterial spectrum while reducing the antibacterial doses (Srichok et al., 2022). Maocheng et al. (2022) investigated the effect of quercetin on induced inflammation of mammary epithelial cells in cattle and investigated its possible mechanism. Yusrizal & Chen (2003) explored how feeding broiler chickens fructan sourced from chicory led to improvements in their weight gain, better feed conversion rates, and lower cholesterol levels. Similarly, Thitaram et al. (2005) verified the beneficial impact of isomalto-oligosaccharide (IMO) supplementation on the gut microbiota of broiler chickens infected with *Salmonella typhimurium*. In another study, Biggs et al. (2007) examined the effects of a diet enriched with five different oligosaccharides, including inulin, TOS, oligofructose, short-chain oligosaccharides, and MOS, on chicken health. Baurhoo et al. (2007) found that excessively high doses of prebiotics can have a negative impact on the gastrointestinal system and slow down the growth of animals (Baurhoo et al., 2007). A review of the literature indicates that studies examining the impact of prebiotics on animal health often yield conflicting results. This inconsistency arises from the unique characteristics of specific compounds, varying dosages, and different administration times. These factors highlight the importance of exploring and utilizing a range of biologically active components as natural strategies to enhance animal performance (Pandey & Rizvi, 2009). The development and use of effective antioxidants of natural origin is a priority nowadays. Medicinal plants can protect animals from free radicals and prevent oxidative damage, which causes degenerative and pathological processes (Tadhani et al., 2007).

In 1995, Gibson & Roberfroid coined the term "synbiotic" to highlight the combined benefits of probiotics and prebiotics working together. They ensure the survival of beneficial microorganisms and stimulate the proliferation of specific local bacterial strains (Markowiak & Śliżewska, 2018). Synbiotics combine the benefits of probiotics and prebiotics, working together to promote the survival of probiotics in our digestive system (Rioux et al., 2005). Nemcová et al. (1999) confirmed the synergistic effect of the bacteria *Lactobacillus paracasei* combined with FOS in the intestinal microbiota of piglets. The researchers observed an increase in the total number of beneficial bacteria of the genus *Lactobacillus* and *Bifidobacterium* in the group of animals fed with the synbiotic. At the same time, the number of bacteria of the genera *Escherichia coli*, Enterobacteriaceae and *Clostridium* decreased in the feces of the studied piglets (Nemcová et al., 1999). Li et al. (2008)

investigated the impact of certain factors on the growth, nutrient digestibility, gas production, and intestinal microbiota composition in weaning pigs. Mohnl et al. (2007) found that the tested product exhibited growth-promoting abilities similar to those of avilamycin in broiler chickens. Meanwhile, Vicente et al. (2007) validated the beneficial effects of a synbiotic product that included *Lactobacillus* spp. alongside lactose. Their research on *Salmonella*-infected turkeys highlighted the positive impact of this symbiotic on both feed conversion and body weight gain. Additionally, Li et al. (2008) explored how administering FOS and the *Bacillus subtilis* bacteria affected broiler chickens, noting enhancements in average daily gain and feed conversion ratios, along with a decrease in the rates of diarrhea and mortality when compared to those treated with tetracycline. Awad et al. (2009) studied the effect of a symbiotic product containing *Enterococcus faecium* bacteria and FOS as a prebiotic, and immunomodulatory substances from seaweed (phycophytes) on the health status of broiler chickens. An increase in the average daily body weight gain, slaughter ratio and feed conversion ratio were found.

In summary, researchers agree that symbiotic products provide better efficacy compared to the separate administration of probiotics and prebiotics (Markowiak & Śliżewska, 2017).

CONCLUSIONS

The use of feed additives, including probiotics, prebiotics, and synbiotics, is both safe and environmentally friendly, while also reducing reliance on antibiotics. However, further investigation is still needed to fully understand the mechanisms behind the actions of these probiotic organisms and their combinations in synbiotics. A wealth of scientific research supports the beneficial effects of probiotics on animal health, including their role in protecting against pathogens, enhancing the immune response, and improving the overall quality of animal production. Additionally, prebiotics can serve as an alternative or complementary approach to boost the benefits of probiotics. The use of a combination of these additives shows a synergistic effect, and can be even more effective in stimulating the intestinal microbiota and improving health. A difficulty in creating synbiotic products is the choice of the appropriate probiotic and prebiotic. Scientific research and development of innovative technologies are needed, which will lead to better production effects and higher quality of animal production.

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THE IMPACT OF SUPPLEMENTING FEED WITH OMEGA-3 FATTY ACIDS ON THE NUTRITIONAL AND TECHNOLOGICAL CHARACTERISTICS OF POULTRY MEAT. A REVIEW

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Abstract

Supplementing feed with omega-3 fatty acid sources is a promising strategy for improving the lipid profile of poultry meat. The study analyzes the effect of diets enriched with flaxseed oil, fish oil and microalgae on the content of polyunsaturated fatty acids, oxidative stability and sensory characteristics of meat. The results indicate a significant increase in the content of EPA and DHA in meat, but oxidative stability was negatively affected, necessitating the use of natural antioxidants to prevent rancidity. These findings highlight the potential of nutritionally enhanced diets to produce healthier meat, but emphasize the need to balance nutritional benefits with product stability.

Key words*: poultry meat, omega-3, polyunsaturated fatty acids, oxidative stability, functional diet.*

INTRODUCTION

In recent years, interest in functional nutrition has increased significantly, as the connection between diet and health has become more evident. Among key nutrients, omega-3 fatty acids - particularly alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) - have attracted considerable attention due to their well-documented benefits on cardiovascular health, cognitive function, and inflammation reduction (Swanson et al., 2012).

Since poultry meat is an important protein source in the human diet, improving its lipid profile through omega-3 feed supplementation becomes a promising strategy for delivering healthier food to consumers. Given the steady yet suboptimal meat consumption in Romania, particularly among women in both urban and rural areas (Pogurschi et al., 2018), enriching poultry meat with omega-3 fatty acids could contribute to addressing common nutritional deficiencies. Nutritional supplementation of poultry feed with omega-3 sources (such as flaxseed, fish oil, or microalgae) has the potential to beneficially alter the fatty acid composition of chicken meat, enhancing its nutritional value (Kralik et al., 2021). Studies indicate that such practices do not negatively impact poultry performance and, in some cases, may even improve bird health via anti-inflammatory and immune-modulating effects (Zelenka et al., 2008).

Furthermore, omega-3 enrichment can contribute to reducing the high omega-6/omega-3 ratio found in modern Western diets, which is linked to an increased risk of chronic disease (Swanson et al., 2012).

MATERIALS AND METHODS

This article is a narrative review that synthesizes current scientific knowledge regarding the supplementation of poultry diets with omega-3 fatty acids and its effects on the nutritional, technological, and commercial characteristics of poultry meat.

The main objective of this review is to summarize and critically assess the current scientific literature regarding: main omega-3 sources used in poultry diets, effects of omega-3 supplementation on meat composition, impact on performance, health, and egg/meat quality, consumer acceptance and technological considerations and the potential of such interventions to contribute to public health via functional animal-based foods.

RESULTS AND DISCUSSIONS

Sources of omega-3 fatty acids in poultry nutrition

Omega-3 fatty acids can be introduced into poultry diets through a variety of sources, each differing in bioavailability, cost, sensory impact, and enrichment efficiency.

The most common plant sources include flaxseed, rapeseed, chia, and their oils. These ingredients are rich in alpha-linolenic acid (ALA), which can be partially converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in poultry tissues (Zelenka et al., 2008).

Fish oil, fishmeal, and microalgae are more direct sources of EPA and DHA and generally offer higher enrichment efficiency compared to ALA-rich ingredients. These long-chain polyunsaturated fatty acids are more readily deposited in meat tissues, with microalgae offering a sustainable and odor-neutral alternative to fish-derived products (Kralik et al., 2021; Elkin & Harvatine, 2023).

In addition to traditional sources, commercial omega-3 supplements - such as encapsulated microalgae oils - are being developed. These formulations improve oxidative stability and allow precise omega-3 dosing in feed, reducing lipid oxidation in both the feed and final meat products (Mane et al., 2024).

A study by Sierżant et al. (2022) demonstrated that flaxseed oil combined with natural antioxidants such as quercetin significantly reduced lipid peroxidation in broiler meat. This combination not only enhanced meat oxidative stability but also preserved omega-3 content without compromising growth performance (Sierżant et al., 2022).

When comparing different plant and marine sources in laying hen diets, Coorey et al. (2015) found that while chia, flaxseed, and fish oil all increased omega-3 content in egg yolk, only fish oil supplementation resulted in measurable levels of EPA and DHA. Interestingly, chia seed supplementation produced a favorable omega-3 profile without altering the sensory quality of eggs, making it a suitable option for consumer-friendly enrichment (Coorey et al., 2015).

To improve the bioavailability of ALA-rich ingredients, recent research has explored nano-encapsulation technologies. Miraeez et al. (2024) showed that nano-encapsulated flaxseed oil led to significantly higher deposition of EPA and DHA in poultry tissues compared to conventional flaxseed oil. Additionally, it upregulated genes associated with fatty acid metabolism, enhancing the nutritional quality of the meat (Miraeez et al., 2024).

The duration of supplementation also plays a critical role. Konieczka et al. (2017) demonstrated that a feeding period of at least two to three weeks with flaxseed, rapeseed, or fish oil is sufficient to achieve meaningful omega-3 enrichment in meat. After three weeks, enrichment levels can meet nutritional labeling requirements in many regions, making short-term strategies cost-effective (Konieczka et al., 2017).

Finally, combining plant and marine-based omega-3 sources has emerged as an effective strategy. According to González-Esquerra & Leeson (2001), dietary blends of flaxseed, canola, and fish oil, along with antioxidant support (e.g., vitamin E), enhanced the omega-3 profile of poultry meat without negatively affecting growth performance or product palatability (González-Esquerra & Leeson, 2001).

Factors influencing the efficiency of omega-3 enrichment

The incorporation of omega-3 fatty acids into poultry meat is influenced by several biological and nutritional variables, including bird species, age, duration of supplementation, and the chemical form of fatty acids. However, genetic background is also a critical determinant.

Recent research emphasizes that genotype plays a key role in how birds respond to dietary interventions. For instance, Tudorache et al. (2022) showed that both genotype and feed composition significantly affect growth performance, carcass yield, and blood biochemical parameters in slow-growing hybrids derived from crosses between local breeds and commercial lines. These findings support the idea that omega-3 supplementation strategies should be tailored to specific genetic profiles to optimize both nutritional benefits and production efficiency.

This highlights the need for genotype-specific nutritional strategies, especially when applying

functional feeds aimed at improving meat quality and health-promoting properties.

Furthermore, fatty acid profile of the supplement determines enrichment efficiency. Long-chain omega-3 sources like fish oil and DHA Gold outperform alpha-linolenic acid (ALA) sources like flaxseed in enriching EPA and DHA in meat tissues (Lemahieu et al., 2015).

In addition, gene expression patterns such as *L-FABP* (fatty acid binding protein), *PPARA*, and *LPL* have been shown to respond significantly to dietary levels and durations of omega-3 supplementation. Higher DHA intake (4.5% tuna oil) over longer periods increases EPA and DHA tissue levels while reducing the n-6/n-3 ratio (Khosinklang et al., 2023).

Another derivative namely nano-encapsulated flaxseed oil showed significantly better tissue deposition of omega-3 compared to its conventional form, due to enhanced intestinal absorption (Miraeez et al., 2024).

A minimum of 2-3 weeks of supplementation is required to see meaningful deposition of omega-3 in tissues, with optimal outcomes around 4-6 weeks before slaughter (Konieczka et al., 2017).

High levels of dietary linoleic acid (omega-6) reduce conversion of ALA to longer-chain omega-3s by competing for desaturase and elongase enzymes (El-Zenary et al., 2020).

Vitamin E plays a critical role in preventing lipid oxidation and preserving omega-3 integrity in enriched meat products (Roux et al., 2011).

Slow-growing chicken genotypes have shown higher deposition of long-chain omega-3 fatty acids compared to fast-growing commercial lines, suggesting species-specific enrichment efficiency (Costa et al., 2017).

Poultry reared under enriched environments (light modulation, perches, tactile stimuli) exhibit improved physiological status and better nutrient assimilation, which can enhance the effectiveness of omega-3 incorporation (Campbell et al., 2019).

Effects of omega-3 fatty acid supplementation on the nutritional composition of poultry meat

Poultry diets can be supplemented with omega-3 fatty acids from plant sources (such as flaxseed or rapeseed oil), marine sources (fish oil, fishmeal), or microalgae. Studies show that flaxseed significantly increases the level of alpha-linolenic acid (ALA), with a partial conversion into EPA and DHA in the meat (Oliveira et al., 2021). However, marine sources are superior in directly increasing DHA levels in poultry tissues (Rymer & Givens, 2005). Baseline differences in fatty acid composition and nutritional value between poultry species have also been reported, suggesting that species-specific considerations may influence the extent of nutritional enhancement through dietary strategies (Costache et al., 2019).

Omega-3 supplementation leads to a significant increase in the content of unsaturated fatty acids in poultry meat. Several studies report a dose-dependent relationship between dietary omega-3 levels and the concentration of EPA and DHA in breast and thigh muscles (Lakshani et al., 2016). The use of microalgae-based feed ingredients is particularly effective in increasing DHA levels in meat without compromising animal performance (Moran et al., 2018).

The impact of dietary interventions on meat quality and bird physiology is often influenced by genotype. For example, Tudorache et al. (2022) demonstrated that both genotype and diet significantly affect performance traits, carcass characteristics, and blood profiles in slow-growing hybrids derived from local and commercial poultry lines. This suggests that omega-3 supplementation strategies may need to be tailored based on the genetic background of the birds.

The efficiency of omega-3 incorporation into poultry meat is influenced by several factors: the type of omega-3 source, bird species, age, duration of supplementation, and the chemical form of the fatty acids. Plant-based sources tend to be less efficient than marine sources in elevating DHA content in meat, mainly due to limited enzymatic conversion from ALA to long-chain PUFAs (Rymer & Givens, 2005; Custură et al., 2024). Baseline differences in fatty acid profiles between poultry species may also influence the potential for omega-3 enrichment, as shown by comparative analyses on eggs and egg products from various bird species (Usturoi et al., 2021).

Omega-3 enrichment may increase the susceptibility of meat to lipid oxidation, which can negatively impact flavor, odor, and shelf life. To mitigate this, antioxidants - either

natural (e.g., tocopherols, rosemary extract) or synthetic - are commonly added to the diet to preserve product quality during processing and storage (Fraeye et al., 2012).

The typical omega-6 to omega-3 ratio in conventional poultry meat is often higher than ideal. Enrichment can lower this ratio from 15:1 to below 4:1, aligning better with nutritional guidelines and contributing to reduced inflammation risks in humans body (Qi et al., 2010).

Most studies show that omega-3 enrichment does not significantly alter the protein, vitamin (e.g., B-complex), or mineral (e.g., iron, zinc) content of poultry meat (González-Esquerra & Leeson, 2001).

However, some variations in antioxidant levels, especially vitamin E (used to prevent lipid oxidation), may occur depending on the enrichment method. Studies show that as the dietary PUFA level increases, vitamin E concentration in tissues decreases unless supplementation is adjusted accordingly. For every gram of dietary PUFA, 2.5–3.7 mg of extra vitamin E may be required to maintain oxidative stability (Barroeta, 2007).

Despite higher feed costs, omega-3 enriched poultry has market potential, particularly in health-conscious consumer segments. Clear labeling and consumer education improve acceptance and willingness to pay a premium price (Barroeta, 2007).

Omega-3 enrichment enhances the nutritional quality of poultry meat while maintaining bioavailability and acceptable sensory traits, offering health benefits and commercial appeal (Gheorghe et al., 2022).

In addition to omega-3 fatty acids, other dietary strategies such as probiotic supplementation have been explored to support gut health and improve performance in broiler production systems (Custură et al., 2019). Such complementary approaches may act synergistically to enhance both animal welfare and product quality.

Technological and Physicochemical Properties of Omega-3 Enriched Meat

The enrichment of poultry meat with omega-3 fatty acids influences several technological parameters, including meat color, pH, and water-holding capacity. The inclusion of omega-3 sources such as fish oil, flaxseed, or microalgae often leads to paler meat due to increased myoglobin oxidation, which affects consumer appeal (Le et al., 2018).

The pH of omega-3 enriched meat may be slightly reduced, altering protein structure and reducing water-holding capacity. These changes can result in drier meat with lower juiciness and reduced yield during processing (Zhi et al., 2023).

Omega-3 enrichment can alter meat texture by softening the muscle fibers and reducing firmness. These effects are dependent on the source and form of omega-3 fatty acids used. Pre-emulsification of oils before incorporation helps maintain a better texture and more uniform structure in processed meat products (Zhi et al., 2023).

Meat products enriched with omega-3 tend to have lower cooking yields if not properly stabilized. Emulsification techniques improve the distribution and retention of omega-3 oils during thermal processing. Pre-emulsification of plant oils helps reduce water and fat losses and improves the structural integrity of products like sausages or meatballs (Zhi et al., 2023).

The enrichment of poultry meat with omega-3 fatty acids significantly influences its sensory profile. Although omega-3 fats are beneficial for health, they can produce unpleasant fishy odors and flavors due to lipid oxidation, especially when fish oil is used (Lee et al., 2023).

Vegetable sources of omega-3, such as flaxseed or canola oil, tend to have a more neutral impact on flavor while maintaining desirable meat juiciness and texture (Le et al., 2018).

Omega-3 fatty acid oxidation leads to the development of volatile compounds that negatively affect taste and odor. This remains a major limitation in the promotion of omega-3 enriched poultry products, as metallic and rancid flavors may appear, especially with prolonged storage (Tura et al., 2024).

Natural antioxidants and microencapsulation technologies can help minimize these sensory issues.

Consumer acceptance is largely influenced by the balance between health benefits and sensory appeal. Studies show that while many consumers are interested in omega-3 enriched foods, taste and odor are critical determinants of purchasing decisions (Lee et al., 2023).

Health benefits of consuming omega-3 enriched poultry meat

Omega-3 fatty acids, particularly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), are essential for maintaining cardiovascular, cognitive, and anti-inflammatory health. Given the decline in fish consumption and the need for alternative sources, omega-3 enriched poultry meat is emerging as a promising dietary option.

Omega-3 enriched poultry meat offers an effective way to supplement essential fatty acids, especially for individuals who do not regularly consume fish. This meat can provide enhanced levels of EPA and DHA, helping to meet daily intake recommendations (Konieczka et al., 2017).

Regular consumption of omega-3 enriched poultry meat may support cardiovascular health by lowering blood triglyceride levels and reducing the risk of heart disease (Brennan et al., 2016).

DHA plays a critical role in brain development and cognitive function. Increased intake through enriched poultry meat may positively impact memory, learning capacity, and help prevent cognitive decline (Brennan et al., 2016).

Omega-3 fatty acids are well-known for their anti-inflammatory effects. Their incorporation into poultry meat may help reduce chronic inflammation and support overall health (Konieczka et al., 2017).

Omega-3 enriched products are generally well accepted by consumers, especially when the meat retains good sensory quality and freshness. Many consumers are drawn to these products for their perceived health benefits (Rymer & Givens, 2005).

Although multiple omega-3 sources have been used in poultry diets (flaxseed, fish oil, microalgae), their efficiency varies significantly depending on the chemical form of the fatty acids and the supplementation duration. A recent study showed that supplementing with the DHA-rich microalga *Aurantiochytrium limacinum* during only the finishing period (21 days) was just as effective as feeding it throughout the entire lifespan, opening the door to more cost-effective strategies (Keegan et al., 2019).

Recent studies have examined how tuna oil supplementation affects the expression of genes involved in lipid metabolism (e.g., L-FABP, PPARA, LPL), showing a clear link between dietary composition and the genetic regulation of omega-3 deposition in meat (Khosinklang et al., 2023). More research is needed to fully understand these mechanisms and their implications for meat quality.

While omega-3 enrichment is nutritionally effective, it may lead to undesirable sensory changes and oxidation. Advanced technologies such as nano-encapsulation of flaxseed oil have shown promise in improving oxidative stability and bioavailability without negatively affecting flavor (Miraeez et al., 2024). Future research should focus on scaling up these technologies for industrial use.

CONCLUSIONS

This review highlights the considerable potential of omega-3 fatty acid supplementation to enhance the nutritional and functional quality of poultry meat. Numerous sources, including flaxseed, fish oil, microalgae, and chia, offer varying levels of effectiveness depending on their chemical structure, bioavailability, and the length of supplementation. Among them, marine sources such as fish oil and microalgae consistently show superior enrichment of EPA and DHA in poultry tissues, while novel delivery methods like nano-encapsulation improve the stability and absorption of plant-based omega-3 sources.

The enrichment process is highly multifactorial. Biological factors such as bird genotype, age, and sex interact with nutritional components - including the type and combination of omega-3 sources, the omega-6/omega-3 dietary ratio, and antioxidant support (e.g., vitamin E) - to influence enrichment efficiency. Environmental conditions and animal welfare also play a role, as enriched rearing systems improve physiological responses and nutrient assimilation.

From a nutritional perspective, omega-3 enriched poultry meat presents improved fatty acid profiles with lowered omega-6/omega-3 ratios, aligning better with dietary guidelines and potentially reducing inflammation risks in consumers. Importantly, protein, vitamin, and mineral contents remain largely unaffected, preserving the meat's baseline nutritional value.

Technological and sensory challenges persist. Omega-3 enrichment may negatively impact

meat texture, water-holding capacity, and flavour - particularly when fish oil is used - due to increased lipid oxidation. However, these effects can be mitigated through pre-emulsification techniques, dietary antioxidants, and the strategic use of more neutral sources like flaxseed or canola oil.

Consumer acceptance of omega-3 enriched poultry meat hinges on sensory appeal, clear health communication, and proper labeling. While omega-3 products can command premium prices, success depends on delivering both health benefits and acceptable eating quality.

Overall, omega-3 enrichment in poultry diets is a scientifically validated strategy for producing functional meat products. It offers a sustainable, accessible way to improve public intake of essential fatty acids, particularly in populations with low fish consumption. Future research should focus on optimizing enrichment protocols by considering genetic responses, improving sensory outcomes, and expanding scalable technologies like microencapsulation for commercial implementation.

Recent trends in poultry meat production emphasize both technological innovation and alignment with consumer expectations for healthier products, which supports the development of omega-3 enriched meat (Moise et al., 2024). Economic feasibility and environmental impact assessments are needed to ensure large-scale adoption (González-Esquerra & Leeson, 2001).

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ANALYSIS OF MILK PRODUCTION EVOLUTION IN THE COW FARMS FROM ROMANIA - NECESSARY MEASURES TO INCREASE THEIR COMPETITIVENESS

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Abstract

In the context of the European single market, a competitive and sustainable increase of dairy farms in Romania is essential for securing their activity continuity and long-term development. Between 2015-2024 the evolution of dairy cows in Romania (number, production yield, quality) was analysed compared to the number and size of farms and farmers' age structure. Dynamics of total national milk production vs milk for processing and EU milk production was evaluated. From income perspective, evolution of raw milk supply prices in Romania (source Milk Market Observatory of European Union) was analysed vs European Union market and compared to price evolution for main feed categories. The results were compared to the dynamics of similar indicators from Poland, a country in Central & Eastern Europe with a remarkable development of milk production. The main conclusions regarding the current level of competitiveness of milk production in Romanian cow farms were highlighted, respectively the long-term risk factors for the national activity of milk production and processing. The necessary measures and related technical-economic levers to consolidate their long-term performance were expressed.

Key words*: competitiveness, measures, risks, production, yields.*

INTRODUCTION

Romania's admission to the European Union (EU) since 2007 represented an important opportunity to access its unique market without trade barriers. This opportunity also came with a strong need for Romanian producers and products to be competitive and economically feasible, beyond the mandatory request to completely comply with EU regulations (e.g. product quality standards). The area of economic activity we have in focus is the agricultural activity and specifically the cow milk production as essential raw material for dairy products.

By analysing the evolution in the period between 2015-2024 of the main technical parameters associated to cow milk production, the main objective is to identify the potential key success factors and main risk categories for the cow milk production in Romania and implicitly, the potential most relevant categories of improvement to be recommended - as a leverage to enhance the competitiveness of Romanian milk production on EU market on a medium/long term.

MATERIALS AND METHODS

The first step of the analysis is to evaluate the degree of development between 2015 and 2023/2024 (based on available public data) for the main parameters of cow milk production in Romania (national milk production, milk production per cow, fat & protein milk content, number and size of the agricultural exploitations based on hectares and cow number, milk price, diesel or fuel price, price for different categories of animal feed associated to dairy cow feeding). Only public data was statistically processed, the main data sources being represented by the Romanian National Institute of Statistics and the European Commission historical databases. The main results were compared with the similar categories of data (for the same period) regarding the milk production evolution in Poland (data collected from Polish National Institute of Statistics and the European Commission historical databases). The selection of Poland for this comparison was made owing the fact that this country switched from a communist system to a democratic system hence from a centralized economy to a market

economy relatively in the same period as Romania (1989), also considering the relatively similar moment of being granted EU membership (2004 for Poland vs 2007 for Romania). At the same time, the similarity of the main geographical characteristics for agricultural land supported this decision. The collected data for the mentioned period was analyzed by calculating the average of the period and the standard deviation, the difference (in percentage) between the beginning and the end of the period. Both national averages were compared between each other and then compared all together with EU average.
The main reached conclusions are the basis to classifying the potential weaknesses and main types of risks associated to Romanian cow milk production efficiency. Implicitly, a set of measures that could enhance on medium/ long term the competitiveness of Romanian milk production was recommended.

RESULTS AND DISCUSSIONS

Milk production (quantity and quality)

For the period 2015-2023 the EU cow milk production registered a continuously yearly growth except for 2020 and 2021, with an average yearly pace of +0.6%, from 147,095 thousand tons to 155,100 thousand tons. In 2020 the milk production decreased with ~1% vs the previous year and remained in a relative plateau in 2021. The highest part of EU milk production (93.88%, as yearly average rate for same mentioned period) was allocated for milk processing (Eurostat, 2024, Milk and milk products).
The Romanian cow milk production decreased with 9.2% during the above-mentioned period from 3,981 thousand tons to 3,611 thousand tons. The share of Romanian milk production from total EU milk production slowly decreased from 2.71% in 2015 to 2.37% in 2023. The Romanian cow milk production refers only to the milking milk production without taking into the amount of milk directly consumed by the calves.
The analysis of available data for Romania emphasizes that this component represents yearly ~9.5% from milking milk production (The National Institute of Statistics of Romania, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2023, 2024, Livestock and animal production).
It is important to underline that Romania has a high specificity among all EU countries concerning the share of milk which is delivered for processing. Compared to EU average share of 93.88% milk from processing mentioned above, Romania has a degree of utilization for processing of only 28.65% from the national milk production with a positive yearly growth rate, from 23.1% in 2015 to 31.1% in 2023 (The National Institute of Statistics of Romania, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, Livestock and animal production. Eurostat, 2024, Milk and milk products).
Implicitly, the share of milk for direct sales, as milk for consumption or dairy products) on a short supply chain (e.g. agricultural markets, fairs) and for self-consumption is strongly higher than for milk allocated for processing.
We may consider that this extreme high share of milk which is not available for processing represents a disadvantage for both milk farmers and milk processors from Romania, especially in those periods when national milk demand for processing is raising above the available milk supply in Romania (temporary increased demands or due to seasonal milk availability). As effect, the shortage in raw milk quantities is covered via import of raw milk or import of dairy finished goods.
Even though the share of milk for direct sales or self – consumption is so high, in practice only very limited quantities of milk could migrate on a very short term to processing due to different reasons: milk quality level (physical – chemical and microbiological), the geographical dispersion of these additional volumes, the associated logistics costs required by the collection, the stability of availability and delivery for processing. During the process of data evaluation, we also analyzed whether a part of this milk on a medium long term could efficiently migrate towards processing and become attractive for processing should the national milk demand increase, for example as an effect of increasing consumer demand. Our perspective will be shared within the conclusions, after the presentation of the results from the evaluation of all data categories.
The milk production was also studied focus on fat and protein content, as main milk quality parameters with direct influence in the yields level of production processes for dairy products.

At EU level, the weighted average of fat content was mentioned as confidential in the public EU reports and the data was available only for 2015-2018. The average fat content is 4.04% with limits ranging between 4.01 and 4.06%; average protein content is 3.43% with yearly increasing trend - from 3.36% to 3.47%.

For the complete period 2015-2023 the fat and protein content for the milk produced in Romania is below the EU average: 3.80% fat and 3.27% protein; we shall underline that both indicators have a positive and steady positive development, from 3.75% to 3.80% the fat and from 3.26 to 3.30% the protein (Eurostat, 2024, Fat and protein cow's milk content, annual data).

The average fat content for the Romanian milk was 3.80% with a standard deviation of 0.0064 pp. Similarly, average protein content for Romanian milk was 3.27% with a standard deviation of 0.0040 pp.

The development of both parameters has a major role because they positively influence the specific consumption of dairy products by decreasing it. This could the effect of the consistent investments made by the Romanian farmers in technology and *know-how*, in the cow genetic value and feed quality. Due to the distinct level of average milk fat and protein content in different EU countries, different fat and protein standards exist for the trading of milk raw; for the levels above standards bonifications are paid.

Evaluating the evolution of Polish milk production between 2015 and 2023 showed a continuously yearly development, from 13,236 thousand tons in 2015 to 15,478 thousand tons in 2023, in percentage from 9% to 10% from the EU milk production.

Assessment of Polish milk production growth rate shows a plus of 16.94% with an average yearly pace of 1.88% (Statistical Yearbook of the Republic of Poland, Statistical Yearbook of Agriculture, 2024); this is much higher than EU average yearly pace of +0.6%, whereas in Romania there was a downward trend of cow milk production quantity. More than 99% from the total milk production is allocated to milk processing, above EU average of 94% and farther away than Romanian milk allocation to processing of 28.35% (Statistical Yearbook of the Republic of Poland, Statistical Yearbook of Agriculture, 2024).

The dynamics of milk production in Romania and Poland for the period 2015-2023 (as percentage from EU total milk production) is presented in the Figure 1.

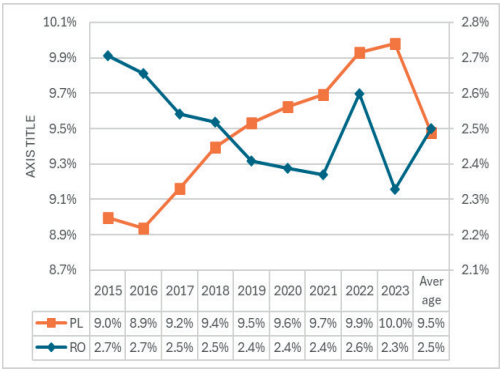


Figure 1. Evolution of Romanian and Polish milk production in the period 2015-2023 (percentage from EU milk production)

The similar evaluation of the progress for fat and protein milk content emphasized a consistent improvement of both indicators, to 4.08% in 2023 (+0.11 pp. vs 2015) and to 3.37% in 2023 (+0.14 pp vs 2015).

For the period 2015-2023 the average fat content for the Polish milk was 4.01% with a standard deviation of 0.0166 pp. Similarly, average protein content for Polish milk was 3.27% with a standard deviation of 0.017 pp (Eurostat, 2024, Fat and protein cow's milk content, annual data).

Both indicators are still under EU average level (presented before) with the fat content close to fulfill the EU average and protein content still relevantly below. The comparison with fat and protein content from Romanian milk shows both indicators are below Polish performance, with a relevant difference for fat. The detailed dynamics is presented in the Figure 2.

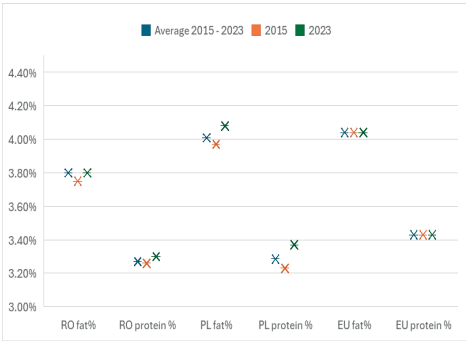


Figure 2. Fat and protein milk content (%) in Romania and Poland (vs EU average), 2015-2023

Therefore, it can easily be stated that Polish milk production has a robust and steady growth, both in terms of quantity and physical and chemical parameters. The yearly rate was higher than EU average for milk production quantity. The increasing fat and protein milk content gets closer to the EU average, the latter being an excellent result which, combined with the quantitative production growth trend, makes milk Polish market an important player of EU milk market.

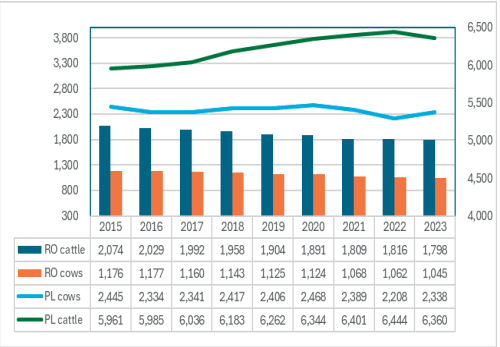
Cow number and yields/cow

The EU dairy cows number decreased during 2015-2023 with 9.9%, from 21,268 to 19,693 thousand heads. At the same time the yield per cow increased with 15%, from 6,760 to 7,970 kg milk (Eurostat, 2025, Number of dairy cows).

The combination of both results increased the milk production competitiveness and emphasizes farmers' focus on higher yields per cow and lower the general costs/cow. The evolution of Romanian number of cattle, out of which the dairies cow number showed a decreasing trend for both categories during same evaluated span of time. Total number of cattle decreased with 13.3% in 2023 vs 2015 and dairy cows number decreased with 11.2%. The share of Romanian dairy cows from the EU similar category slowly decreased from 5.4% to 5.3% (The National Institute of Statistics of Romania, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, Livestock and animal production. Eurostat, 2024, Milk and milk products).

In Poland, compared to Romania, the cattle total number increased with 6.5%; the dairy cows number decreased with 4.4%, but with a slower pace than EU average. Implicitly, the share of Polish dairy cows from EU similar category slowly increased from 11.3 to 11.8% (Eurostat, 2025, Number of dairy cows). To sustain the already discussed data another chart regarding progress of total cattle number, out of which dairy cows, in Romania and Poland, 2015-2023, is presented below (Figure 3).

The milk production per cow increased in EU, Romania and Poland, the only difference being the pace at which these expansions happened. EU yield per cow grew with 15.25% in 2023 vs 2015 vs only +2.45% in Romania and an excellent 24.20% in Poland.

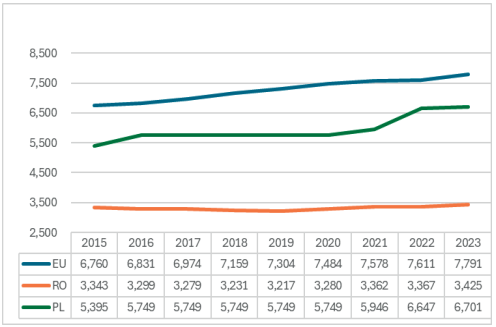


	2015	2016	2017	2018	2019	2020	2021	2022	2023
RO cattle	2,074	2,029	1,992	1,958	1,904	1,891	1,809	1,816	1,798
RO cows	1,176	1,177	1,160	1,143	1,125	1,124	1,068	1,062	1,045
PL cows	2,445	2,334	2,341	2,417	2,406	2,468	2,389	2,208	2,338
PL cattle	5,961	5,985	6,036	6,183	6,262	6,344	6,401	6,444	6,360

Figure 3. Cattle and dairy cow number in Romania and Poland, 2015-2023 (thousand heads)

As effect, Poland recovered a part of the gap vs EU average: in 2015 the average performance of Polish milk cow was 79.8% from EU average cow performance while in 2023 represented 86%. On the other hand, in Romania the gap increased with 5.5 pp, because in 2015 the average performance of a Romanian milk cow was 49.5% and decreased up to 44.5% from the average performance of an EU milk cow (European Commission, 2024, Data-Modelling platform of resource economics, EU-27 estimated agricultural balance sheets).

The detailed evolution is presented in Figure 4.



	2015	2016	2017	2018	2019	2020	2021	2022	2023
EU	6,760	6,831	6,974	7,159	7,304	7,484	7,578	7,611	7,791
RO	3,343	3,299	3,279	3,231	3,217	3,280	3,362	3,367	3,425
PL	5,395	5,749	5,749	5,749	5,749	5,749	5,946	6,647	6,701

Figure 4. Milk production/ cow (kg) in EU, Romania and Poland (2015-2023)

Pursuant to evaluating in conjunction all categories of data mentioned before - number of dairy cows, milk production/cow, fat/ protein content - we can conclude that even though Romania owns 5.3% from total number of dairy cows, it covers only 2.37% from total EU cow milk production, in decreasing trend.

Fat and protein content are still primarily below EU average – even if they registered a growth. Although there is no sense in denying the importance of yield per cow growth, the rising rate has yet to reach the EU average yield/cow.
We also must accentuate that the average milk production per cow is calculated at national level considering the total number of dairy cows, while it was mentioned before that in Romania only 30% of the total milk production is dedicated to processing.
The connection between these two aspects includes also the fact that the milk production from commercial farms which deliver their milk for processing is continuously under a consistent and important optimization process to remain competitive on the raw milk market. At the same time, the milk production from the agricultural exploitations with a very low number of cows (below 5 cows) has a much lower production performance. A higher part of these milk volumes is not directed to processing but kept for self-consumption or selling on a short supply chain.
The evaluation of milk data for Polish milk production and cow numbers shows that Poland can be considered an example for the East and Central part of Europe. All the analyzed parameters support the development of milk production in term of quantity and quality and the production performance per cow.
The milk volumes availability and increasing milk quality development enhance Polish position as a reliable milk source alternative on EU milk market.

Agricultural exploitations (number and size)

In 2020 the number of EU agricultural exploitations was 9,070 thousand units, out of which 2,887 thousand units in Romania (31.83%) and 1,302 thousand units in Poland (14.36%) (Eurostat, 2024, Farm indicators by legal status of the holding, utilized agricultural area, type and economic size of the farm).
Romania was responsible in 2020 for approximately one third of total number of agricultural exploitations but regarding the outcomes only for 3.3%. The 2020 structure of agricultural exploitations based on number of ha per exploitation reflected that EU has close to 64% exploitations below 5 ha, while RO was above 90% (90.32%) vs Poland with a better status of 53% exploitations below 5 ha (Eurostat, 2022, Farms and farmland in the European Union- statistics).
The detailed evolution is presented in Figure 5.

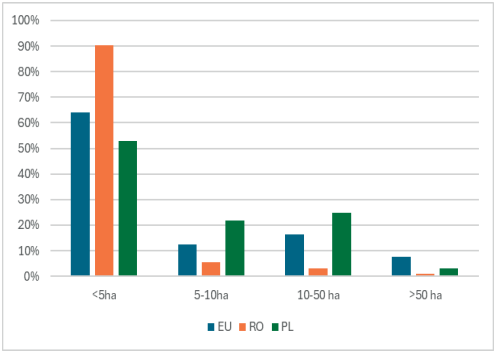


Figure 5. Exploitations structure in hectares (2020)

In Romania, the very high number of agricultural exploitations has a relevant decreasing tendency between 2013 and 2023 - from 3,630 thousand units to 2,859 thousand units in 2023, which means 21.33% less. More than 99% from total number of exploitations represents exploitations without legal personality and with an average size of ~2.5 ha. The number of exploitations with legal personality is only ~25 thousand units, slow decreasing trend; the average size (ha) is consistent, even with a slow decreasing trend, from 206 to 190 ha in 2023; detailed evolution is presented in Table 1 (The National Institute of Statistics of Romania, 2014, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, Agricultural holdings, areas and cattle herds).

Table 1. The number of Romanian agricultural exploitations and average size (thousand units, ha)

Criteria		Agricultural exploitations (x1000 units)	Agricultural land/ exploitation (ha)
2013	total	3,630	3.6
	w/o LP	3,602	2.0
	with LP	28	206.6
2016	total	3,422	3.7
	w/o LP	3,396	2.0
	with LP	26	213.6
2020	total	2,887	4,4
	w/o LP	2,862	2,7
	with LP	25	194,7
2023	total	2,859	4,4
	w/o LP	2,834	2,7
	with LP	25	190,5

Note: LP = legal personality

Evaluating density of cattle per 100 ha during 2015-2023, out of which dairy cows (heifers included) per 100 ha, we noticed a considerable higher density of total cattle in Poland vs Romania: 43 heads in Poland in 2023 vs 41 in 2015, while in Romania only 13.9 heads in 2023 with a slow decreasing tendency vs. 15.6 heads in 2015.

Density of dairy cows/100 ha is much lower in Romania vs Poland, less than 10 heads in Romania vs 16 in Poland, in a relative stagnation; the complete evolution is presented in Figure 6 (The National Institute of Statistics of Romania, 2011, General agricultural census; 2017, Farm structure survey; 2024, Farm structural survey. Statistical Yearbook of Republic of Poland, Statistical Yearbook of Agriculture, 2024).

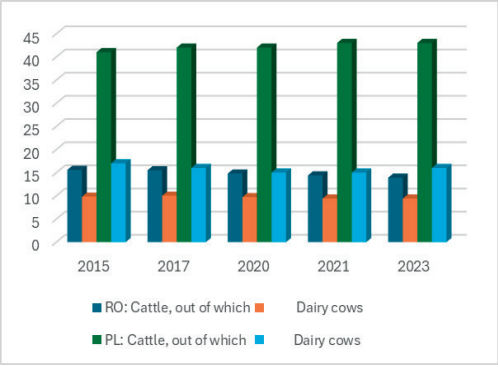


Figure 6. Density of cattle, out of which dairy cows/100 ha in Romania and Poland (heads, 2015-2023)

The evaluation of exploitations structure with cattle in Romania when it comes to size (number of cattle) was done by comparing the available 2010 and 2016 data, while 2023 data are expected to be made public available during first semester of 2025 (The National Institute of Statistics of Romania, 2011, General agricultural census; 2017, Farm structure survey; 2024, Farm structural survey).

The data analysis reflected an extremely high share of exploitations with a very low number of cattle: two thirds of the exploitations with cattle have below 2 heads and close to 30% of exploitation have between 2 and 9 heads. Even if noticed an increasing tendency for number of heads per exploitation the average size (number of cattle) remains extremely low.

The evolution of structure for the agricultural exploitations is presented in Table 2.

Table 2 Structure of exploitations with cattle in Romania

Year/nr of exploitations with cattle	< 2 heads	< 9 heads	> 100 heads	> 500 Heads
2010 (728,000)	73.7%	23.6%	0.08%	0.01%
2016 (541,000)	66.1%	29.5%	0.10%	0.02%

The evaluation of overall categories of data - number of exploitations, with and without legal personality, the density of cows per 100 ha and the size of exploitations in heads of cattle per exploitation, shows that in Romania there is still a high dispersion of total land among agricultural exploitations, the number of cows per exploitation being also very small; on top of that 99% of the exploitation does not have legal personality. We can understand why the milk production competitiveness in Romania as national average is so reduced.

It is also of upper significance to underline the fact that from these small agricultural exploitations, the majority without legal personality, a high share of raw milk is kept for self-consumption or for selling on short supply chain. This stands as an explanation of the fact that only ~30% of total national milk production is transferred for processing.

It is a low probability for these milk volumes (coming from very small milk cow exploittations) to continue to exist on a long term at the same volume scale and to migrate in a stable and competitive way for processing in dairy products.

This equation is even more sensitive when we consider in evaluating the above-mentioned aspects the fact that ~40% from the total number of Romanian agricultural exploitations are owned by people of 65 years or more (Eurostat, Farmers and the agricultural labour force – statistics – Statistics Explained, 2022).

Milk price and feed price dynamics

The next column finds its aim into analyzing the milk price between 2015 and 2024 (as 2024 was already publicly available). The milk price was evaluated for comparison with EU average price and between both countries. The reported prices are expressed as farm gate prices, in eurocents per kilogram and considering different fat and

protein milk content, in accordance with reported national average for both parameters.
The dynamics of milk price was also compared with the evolution of diesel price (using the same methodology), considering the relevance and impact of diesel cost in the entire milk production cost. Likewise, the evolution of price for corn kernel was evaluated on both markets to have a comparative image about the development of this raw material for the same period.
For the Romanian market, the milk price development was also evaluated comparatively to the evolution of price for green corn for silage, with respect to its relevance as cost driver in the feed ratio for milk production.
The EU cow milk price strongly increased between 2015 and 2024, with ~60%, from a level of 30.34 to 48.41 eurocents/kg. EU average price was 37.98 eurocents per kg with a standard deviation of 7.69 eurocents. The peak milk price was in 2022 at 50.21 eurocents/kg.
The Romanian milk price for cow milk increased (in percentage) stronger than EU average rate for 2015-2024, with 71.47%, from 26.32 to 45.12 eurocents/kg. Romanian average price was 34.46 eurocents per kg with a standard deviation of 8.49 eurocents. Peak milk price was in 2022 at 47.44 eurocents/kg.
The Polish cow milk price showed the strongest progress between 2015 and 2024, with 77.47%, from 27.83 to 49.51 eurocents/kg. Average Polish price was 36.01 eurocents per kg with a standard deviation of 8.73 eurocents. Peak milk price was in 2024 at 49.51 eurocents per kg, followed by 2022 price of 49.01eurocents per kg (European Commission, 2025, Milk market observatory - dashboard).
Both Romanian and Polish prices were able to decrease their gap vs EU average price, the Romanian price with a lower amplitude than Polish price. As effect, compared to the EU average price of 37.98 eurocents per kg, the Romanian price was lower with 9.26% vs the gap of 5.16% for the Polish milk price. Evaluating RO average price vs Polish average price for cow milk, we may say that for the period 2015-2014 the Polish average price was higher with 4.52% than Romanian price.
Corroborating the image presented before regarding the fat and protein milk content, the milk production per cow and the average size of cattle exploitations together with dynamics of milk price, we may say that the increasing milk production performance in Poland is also reflected in the milk price development, with a stronger rate than EU milk market price increase (European Commission, 2025, Milk market observatory - dashboard). The detailed dynamics is presented in Figure 7.

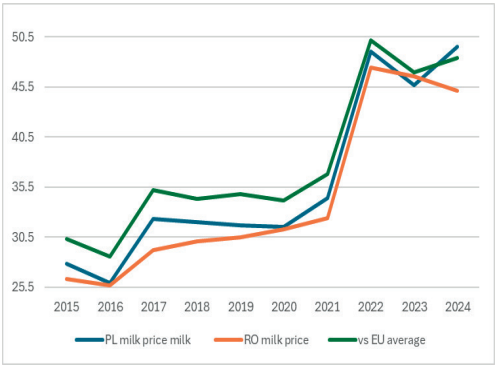


Figure 7. Milk price dynamics in Romania, Poland and EU, 2015-2024 (eurocents per kg)

The evaluation of diesel price shows that at EU level the fuel price strongly grew between 2015 and 2024, with 29.97%, from 1.24 to 1.61 euro/liter. The EU average price was 1.39 euro/l with a standard deviation of 0.235 euro. Peak milk price was in 2022 at 1.82 euro/liter.
The Romanian diesel price increased in percentage lower than EU average rate between 2015 and 2024, with 22.21%, from 1.20 to 1.46 euro/liter.
It is relevant to mention that the starting point in 2015 was a higher price for diesel in Romania vs Poland (1.20 vs 1.08 euro/l). The Romanian average price was 1.24 euro/l with a standard deviation of 0.219 euro. The peak milk price was in 2022 at the level of 1.64 euro/l.
The Polish diesel price had the strongest raise between 2015 and 2024, with 39.03%, from 1.08 to 1.50 euro/l. The average price was 1.21 euro per kg with a standard deviation of 0.214 eurocents. Peak diesel price was in 2022 at 1.54 euro/l.
Comparing to EU average price of 1.39 euro/l, the Romanian price was lower with 10.38% vs EU average price for diesel; the gap between Polish diesel price and EU average prices is –12.94%. Comparing the RO average price with Polish average price for diesel, we may say that

for the period 2015-2014 the Polish diesel price was lower with 2.86% than Romanian price (European Commission, 2025, Weekly oil bulletin).
The evolution of diesel price in EU, Romania and Poland is presented in Figure 8.

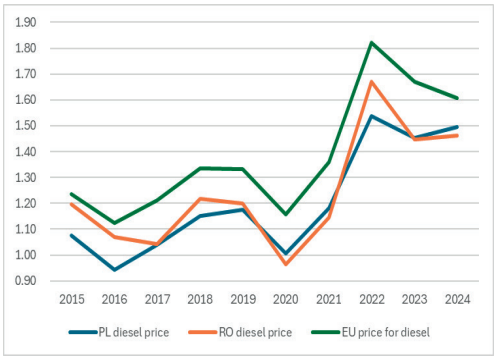


Figure 8. Diesel price development in Romania, Poland and EU, 2015-2024 (euro/l)

The data reflects an important increase in absolute values for the diesel price, even if the gap price for Romania and Poland vs EU price is increasing in percentage.
Comparing Polish diesel price dynamics with the similar one in Romania we may say that the share of costs due to the fuel price is stronger for the Romanian farmers than the Polish farmers in the context where milk price revenues are smaller for Romanian farmers.
The analysis of corn kernel price evolution in Romania shows an important price increase between 2015 and 2024, with 34.79%, from 0.171 to 0.230 euro/kg. Romanian average price was 0.185 euro/kg with a standard deviation of 0.044 euro. Peak milk price was in 2022 at 0.28 euro/kg, followed by 2023 at 0.23 euro/kg.
The Polish price for corn kernel had a decrease of 7.94% between 2015 and 2024, from 0.135 to 0.125 euro/kg. The average price was 0.128 euro/kg per kg with a standard deviation of 0.005 euro.
The peak milk price was in 2015, with yearly decreases until 2023 inclusive (The National Institute of Statistics of Romania, 2025, Vegetable agricultural production, Economic accounts in agriculture; Prices of agricultural products).
Evaluating Romanian average price with Polish average price for corn kernel, we may underline that for 2015-2023 the Polish price was lower with 30.86% than Romanian price.
The results reflected an increasing cost for the Romanian farmers vs Polish milk farmers by using the corn kernel in the recipe of energy concentrated feed for dairy cows. Implicitly, this affects the milk profitability per liter of milk produced, together with the other challenges mentioned before.
The evolution of corn kernel price in EU, Romania and Poland is presented in Figure 9.

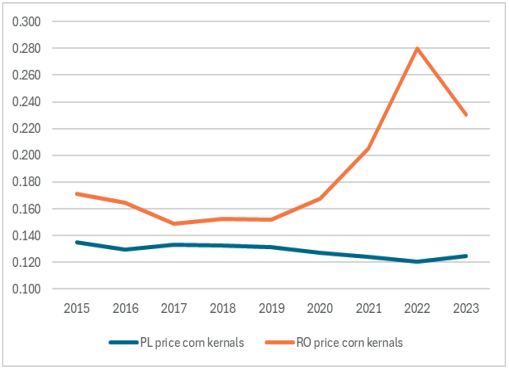


Figure 9. Corn kernel price development in Romania and Poland, 2015-2023 (euro/kg)

The Romanian price for green corn for silage increased in percentage with 7.60% between 2015-2023, with a peak of 38.44% in 2022 vs 2015, from 0.0055 to 0.0076 euro/kg. Romanian average price 2015-2023 was 0.0063 euro/kg with a standard deviation of 0.009 euro. The peak milk price was in 2022 at the level of mentioned before. The detailed price evolution for green corn for silage in Romania is presented in Figure 10.

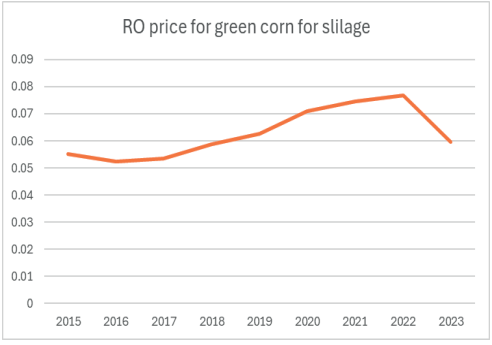


Figure 10. Green corn for silage price development in Romania, 2015-2023 (euro/kg)

The prices for diesel fuel, green corn for silage and corn kernel were for Romanian and Polish markets were converted from the national currency to euro using the yearly parity euro/ron and euro/zloty communicated by each National Bank for the evaluated period (Narodovi Bank Polski, 2025, Historic average exchange rates; National Bank of Romania, 2025, Historic average exchanges rate).

We must accentuate that the impact of increasing prices for cost drivers as diesel fuel, green corn for silage and corn kernel has to be weighted with their corresponding share in the milk production cost, data which were not in the objective of this evaluation. Despite this, the analysis of evolution for all these cost drivers shows that their shares in the milk production cost for the Romanian farmers is increasing at a higher rate than for Polish farmers, which affects the possibility of Romanian milk producers to be competitive vs farmers from other EU countries. In addition, as mentioned before, given that the milk price in Romania is lower that the milk price for Polish farmers, we have a picture of the strong challenges the Romanian milk farmers must manage.

CONCLUSIONS

Summarizing the results and the discussions presented in the previous chapters, the main conclusion is that between 2015 and 2023/2024 the increase of milk production performance in Romania (milk production per cow, fat and protein content, average size of agricultural exploitation with cattle and number of ha/exploitation) cannot enhance the needed efficiency for the Romanian milk farmers.

This perspective has to be considered in a context in which the milk price index is enlarging its gap vs the EU average milk price level. The increase of the milk production cost drivers is affecting even stronger the profitability of the Romanian milk producers in a context we may see higher increasing price levels for them in Romania vs other countries, such as Poland.

The development of Polish milk production could be considered an example from which the positive aspects are worth to be identified and replicated, if possible, in Romania as well.

Taking in mind the unique EU market for cow milk production and milk marketing, the need for technical and economic efficiency of the Romanian milk production is essential to secure the availability of Romanian milk market on a long term. This last aspect should be considered within the frame where the high volumes of existing Romanian milk which are now kept for self-consumption or selling on a short supply chain, have a low possibility to migrate towards milk processing and to become competitive, both technically and economically.

Strictly respecting the existing EU regulations, it might be recommended that the enhancement of those State measures which could support a stronger improvement of Romanian milk production efficiency to be also evaluated at national level: land concentration in the favour of young farmers, higher accessibility to financial resources guaranteed by the State, fiscal facilities to encourage the long term investments in the genetical value of the cows, in land concentration and for animal husbandries technologies.

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EFFECTS OF BEHAVIOUR DURING MILKING ON PRODUCTION AND REPRODUCTION INDICATORS IN ROMANIAN WATER BUFFALOES

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Abstract

The aim of this study was to evaluate the effects that behaviour during milking has on production, reproduction and welfare indicators in water buffalo cows reared under loose housing system. The study was conducted in a private farm, on 102 dairy buffalo cows (6.4±0.1 lactations) at the beginning of their lactation (100 days in milk, DIM). The behaviour during milking was evaluated using a 5-point subjective scale (1 – calm to 5 – nervous), using two individual trained observers placed at 0.5-1 m behind the animals, using an 8x2 herringbone milking parlour. The animals were grouped based on their behaviour as 'calm' (scores 1, 2 and 3; n = 76) or 'nervous' (scores 4 and 5; n = 26). Milk yield and milking speed were significantly influenced by the behaviour during milking (p>0.05), with calmer buffaloes outperforming their nervous counterparts. No significant influence of the behaviour during milking was found on body condition score, calving interval, age at first calving or animal-based welfare indicators. Current findings suggest that incorporating behavioural assessments during milking into management practices could enhance productivity in dairy water buffaloes.

Key words*: animal-based indicators, animal welfare, milking behaviour, water buffaloes.*

INTRODUCTION

Comparative neuroanatomical analyses and studies of emergent cognitive–behavioural functions in human ancestors suggest that temperament is linked to associative conditioning, a trait highly conserved across all species (Cloninger et al., 2009). Studying temperament (behavioural reactivity) provides a direct insight into the adaptive mechanisms that evolved in animals, which allowed them to quickly and effectively respond to external stimuli, being essential for their health and survival (Cloninger et al., 2019).

Different temperaments evolved to help animals adapt to naturally occurring events, with variations in reactions to both external and internal stimuli being documented. These variations were essential regulation of the immune and inflammatory response and energy production maintenance, along with processes that mediate habit learning, emotional reactivity, cognitive flexibility, sensory sensitivity and circadian rhythmicity (Mendoza et al., 2011; Steelman et al., 2011; Dyson et al., 2015).

Livestock behaviour during handling is influenced by both genetics and past experiences, with recent research suggesting that fear is just one of the many temperament traits and proper (gentle) habituation from a young age, especially with feed rewards, can promote calmer animals (Grandin et al., 2024). For large domestic ruminants like cattle and water buffaloes, the social environment plays a crucial role for their well-being (De Freslon et al., 2020), as there is evidence that these species can exhibit aspects of social complexity, such as social learning (Cooke et al., 2021). Water buffaloes (*Bubalus bubalis*) are predominantly reared in traditional production systems world-wide, nonetheless modern intensive systems and mechanization are expanding rapidly in some European countries, such as Italy, Romania and Bulgaria (Borghese et al., 2010). This shift has

inevitably displaced water buffaloes from their natural habitats and brought changes to various aspects of intensive farming, such as feeding, milking machines, handling and housing conditions (De Rosa et al., 2005; Napolitano et al., 2019). While buffaloes are becoming more prevalent in milk production, their temperament and strong bond with their calves make the entire milking process challenging (Erdem et al., 2022). Like all animals, water buffaloes rely on associative conditioning for learning and adapting to their environment, particularly in response to handling, milking and human interactions. However, unlike higher primates and humans, their ability for intentional self-control and self-awareness is limited, meaning that their behaviour is primarily driven by learned associations, sensory experiences and innate temperament traits (Zwir et al., 2020).

Frequent human-animal interactions in dairy species (particularly during milking routine) are closely associated with productive and welfare indicators (Simitzis et al., 2021), with strong evidence (especially in dairy cows), that calmer and less reactive animals outperform their reactive counterparts when milk attributes are taken into consideration (Haskell et al., 2014). In general, temperament describes an animal's individual response to human handling and, when heritable, this trait holds potential for improving welfare through the selective breeding of animals that are less reactive to handling. In dairy water buffaloes, behaviour during milking refers to the animal's response throughout the entire milking process. This behaviour is commonly assessed using visual scoring systems that evaluate the frequency and intensity of movements, which in turn reflect levels of fear and overall responsiveness (Hedlund et al., 2015). Lactating water buffalo cows exhibit a heightened sensitivity to handling during milking in comparison to dairy cattle, a difference attributed to their relatively limited genetic selection for traits associated with milking performance (Thomas et al., 2005; Bidarimath and Aggarwal, 2007). Previous research has emphasized that adequate pre-milking stimulation, together with stressors mitigation during milking routine, are essential for triggering the ejection of the alveolar milk fraction, which constitutes approximately 90–95% of the total milk yield in this species (Costa et al., 2020). In buffalo cows, exposure to even minor environmental or procedural changes during milking can elicit a stress response characterized by elevated adrenaline secretion. This, in turn, suppresses endogenous oxytocin release, thereby impairing the milk ejection reflex (Borghese et al., 2007). Given the limited success of genetic selection in enhancing spontaneous milk ejection in buffaloes, exogenous oxytocin administration has become a widespread practice in large-scale production systems to facilitate milk let-down (Napolitano et al., 2019; Shahid et al., 2021). However, repeated administration of oxytocin has been proved to adversely affect the mammary gland secretory function and to inhibit the physiological milk ejection mechanism (Mustafa et al., 2008). Moreover, it has been associated with detrimental effects on reproductive performance, delaying the onset of a new ovarian cycle and contributing to reproductive disorders such as poor estrus expression, low conception rates, longer calving intervals and increased embryonic mortality in buffalo cows (Faraz et al., 2020).

Given the limited research on the implications of temperament in this species, the overall objective of this study was to evaluate the effects that behaviour during milking has on production, reproduction and welfare indicators in water buffalo cows reared under loose housing system (Noce et al., 2021; Vidu et al., 2013). The hypothesis was that calmer and less excitable buffalo cows would outperform their more reactive and nervous counterparts, when taking into account the production variables, reproduction outputs and welfare indicators.

MATERIALS AND METHODS

Animal management and milking reactivity assessment

The research was conducted in a commercial farm (46°11'77''N 25°1'33''E) in Mesendorf, Romania, (altitude of site 460 m), on 102 (6.4 ± 0.2 lactations) dairy water-buffalo cows. The study focused on animals at the beginning of lactation (first 100 days of lactation, 100 DIM) and was carried out between May and August 2022. Buffaloes were managed under identical

feeding and housing conditions, with a loose housing system and individual resting cubicles, with a space allocation of 10 m² per animal in the resting and movement area. *Ad libitum* access to water, mineral blocks and outdoor paddocks was provided. The feeding system at the farm was not seasonally differentiated, with the fodder being ensured from the farm's own resources. Feed was administered as a total mixed ration (TMR) twice a day in the paddock feeding line, with a feeding space of 90-100 cm per animal. The daily ration per buffalo included 7 kg alfalfa hay, 7 kg pasture hay, 4 kg corn silage and 2.5 kg concentrates. Animals were kept in groups of 50 to 60 animals, depending on their lactation stage and productivity. Reproduction was conducted through natural mating (one bull for each 20-25 buffalo cows), while inbreeding was avoided by replacing the bulls every 24 months. The animals were milked twice daily (starting at 5:30 AM and 5:30 PM) in a "herring-bone" milking parlour (2 x 8 units). The milking parlour was equipped with GEA DemaTron 70 software and hardware and the milking machines were fitted with individual milk meters (MKV Milk Meter). Milking reactivity (MR) was assessed according to the protocol described by Mincu et al. (2022), with two trained observers positioned in the main alley of the milking parlour throughout the entire milking process. Each buffalo cow was scored on a 5-point scale (score 1 = calm response to the milking procedure to score 5 = highly reactive response, in increments of 1). Based on the milking reactivity scores assigned during milking, the buffaloes were classified as either "calm" (scores 1, 2 and 3; n=76) or "nervous" (scores 4 and 5; n=26).

Data collection and welfare assessment

A total of 12 parameters per buffalo were considered to evaluate the effects of behaviour during milking (milking reactivity) on production, reproduction, and welfare indicators. The identification number, milk yield per milking session (kg), and milking duration (minutes) were recorded directly during MR assessment. The milking speed (kg/min) for each animal was obtained by dividing the milk yield by the milking duration. Additional production data were collected, including milk yield per 100 days in milk and daily milk yield per buffalo (kg/day), which was measured twice during the data collection period, following standardized guidelines (ICAR, 2012). The reproductive performance of the studied buffalo cows, including age at first calving and calving interval, was recorded by the farm veterinarian and technicians. Additional welfare-related data were collected during MR assessment day, including body condition score (BCS), cleanliness of udder, rump and hind legs, excessive hoof growth, skin lesions incidence, hairless patches, nasal, ocular and vulvar discharges incidence, and tarsal joint lesions. The BCS of the buffaloes was assessed using the 9-point scale method (where score 1 was severely emaciated, with no presence of fat either visible or palpable, and physically weak, and score 9 was severely obese with typical 'fat pads'), in increments of 1, according to a previously developed scale by Negretti et al. (2008). Tarsal joint and skin lesion incidence were assessed during the behavioural evaluation of the animals. Claw overgrowth, considered an indicator of lameness, was categorized into three levels: 0 - mild growth, 1 - moderate growth, and 2 - severe growth, following the method described by Whay et al. (2003). Integumentary alterations, including hairless patches, were scored as follows: 0 - no hairless patches, 1 - presence of at least one hairless patch, and 2 - multiple hairless patches. Additionally, nasal, ocular, and vulvar discharges were evaluated individually based on the Welfare Quality® (WQ) protocol for dairy breeds, with a score of 0 indicating no visible discharge and 2 indicating the presence of discharge.

Statistical analysis

The data were statistically analysed to estimate the effects that behaviour during milking has on production, reproduction and welfare indicators in water buffaloes raised in an intensive system. The influence of behaviour during milking (calm vs. nervous) on production, welfare and reproductive indicators in buffaloes was analyzed using both descriptive and inferential statistics. Mean and Standard Error of the Mean (SEM) were calculated for all continuous variables. The distribution of Body Condition Scores (BCS)

was compared between reactivity groups using Fisher's Exact Test to assess whether behaviour during milking influenced the proportion of animals in different BCS categories. Logistic regression was applied to evaluate the association between milking reactivity and welfare indicators, with odds ratios (OR) used to quantify the likelihood of poor conditions in nervous animals relative to calm ones. Reproductive traits, including calving interval and age at first calving, were analyzed using the Mann-Whitney U test due to non-normal data distribution. Differences in milking speed and milk yield were assessed using the Wilcoxon rank sum test, providing insights into the impact of milking reactivity on milk production efficiency. Results were visualized using violin plots for reproductive parameters and bar plots with mean ± SEM for BCS distribution and milking traits. Statistical significance was set at $p < 0.05$ and all analyses were conducted using R® software.

RESULTS AND DISCUSSIONS

Significant differences in productive indicators were observed between calm and nervous buffalo dairy cows.

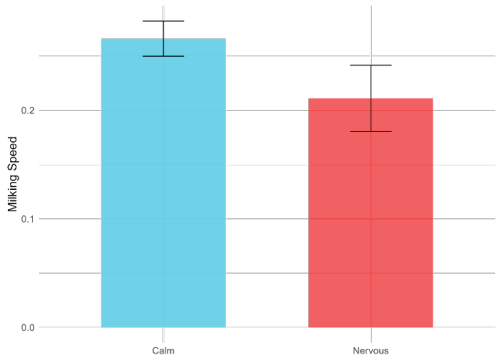


Figure 1. Milking speed (kg/min) for calm and nervous buffaloes (mean ± SEM)

For milking speed, calm buffalos were more efficient, with an average speed of 0.266 kg per minute and lower variability (SEM = 0.0162), compared to 0.211 kg per minute in nervous buffalos, which exhibited higher variability (SEM = 0.0305). The difference was statistically significant ($p = 0.0436$), indicating that calm animals are better suited to the milking process, likely due to reduced stress and better disposition during milking (figure 1). In contrast, the slower milking speed in nervous buffalos might prolong the milking routine, increase labour costs and reduce the overall efficiency.

Milk yield for 100 DIM has showed even more significant diferences. Calm animals produced an average of 399 liters per lactation, compared to 316 liters in nervous cows, with a substantial difference of 83 liters. This disparity, also statistically significant ($p = 0.0436$, Figure 2), has serious economic implications. With current milk prices, such losses could significantly impact farm's profitability, emphasizing the need to address temperament-related traits through improved handling and animal selection practices.

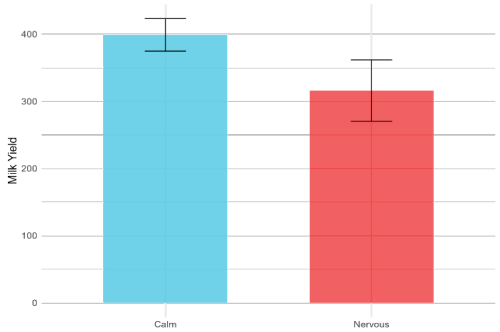


Figure 2. Milk yield (kg/100DIM) for calm and nervous buffaloes (mean ± SEM)

These findings suggest that temperament is a key factor influencing productivity in dairy water-buffalo farming. Calm animals consistently outperformed nervous buffalos, demonstrating higher milking speed and greater milk yields. Current results are in accordance with Erdem et al. (2022), which found that higher milking temperament scores in Anatolian buffaloes were significantly correlated with increased frequencies of stress-related behaviours such as vocalizing, kicking, stepping, defecating, urinating and pulling the teat cup off. Additionally, higher temperament scores negatively impacted milk yield per milking and milk flow rate, while increasing milking time, with primiparous buffalo cows showing higher temperament scores, though these decreased over time as they got habituated to the milking procedures.

The impact of behaviour during milking (calm vs. nervous animals) on reproductive indicators was evaluated by comparing the calving interval and age at first calving between the two groups. Descriptive statistics showed that the mean calving interval was 465 ± 17.9 days in the calm group and 468 ± 26.6 days in the nervous cows. Similarly, the mean age at first calving was 71.0 ± 4.23 months in calm buffaloes and 67.3 ± 6.49 months in nervous animals. The Mann-Whitney U test was used to assess statistical differences between temperament groups for both traits. No significant difference was observed for the calving interval ($W = 887$, $p > 0.05$), indicating that milking reactivity does not play a major role in this reproductive parameter (Figure 3). Likewise, age at first calving did not differ significantly between the two groups ($W = 1055$, $p > 0.05$), suggesting that milking reactivity does not have a measurable effect on the timing of reproductive maturity (Figure 4). These findings indicate that reproductive efficiency in dairy buffaloes is not strongly influenced by temperament related traits.

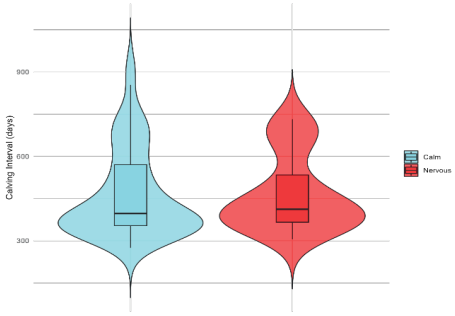


Figure 3. Descriptive statistics (violin plots) for calving interval in calm and nervous buffaloes (*Violin plots display the full distribution of the data through a rotated kernel density plot, where the width of each violin represents the frequency (density) of the data points. The black box inside each violin indicates the interquartile range (IQR), while the horizontal line shows the median value)*

In contrast to the findings of the present study, Khan et al. (2007) reported that calm-tempered buffaloes had shorter calving intervals and higher conception rates, compared to their more reactive counterparts.

The discrepancies between the two studies might be attributed to several factors, the most relevant being the use of different breeds (with the Romanian Buffalo being a rustic breed, not selected for reproductive precocity), environmental conditions (such as climate and altitude), as well as the different rearing and feeding systems.

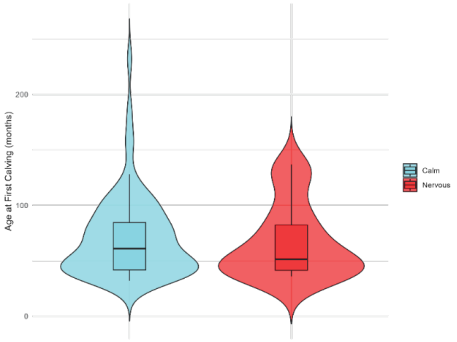


Figure 4. Descriptive statistics (violin plots) for age at first calving in calm and nervous buffaloes

There was no statistically significant difference in the distribution of BCS categories between the calm and nervous groups (Fisher's Exact Test, $p = 0.4741$). Body Condition Score (BCS) was analyzed to assess differences between temperament groups, expressed as the percentage of individuals within each group falling into specific BCS categories. Additionally, Figure 5 illustrates the mean ± SEM for each BCS value across the two groups, providing a clearer picture of the internal variability. In the calm group, BCS values ranged from 4 to 9. The distribution was relatively broad, with a higher concentration in the upper BCS categories. The highest percentage of animals was observed at BCS 8 (34.2%), followed by BCS 7 and 9 (21.1% each). Lower BCS scores were rare: BCS 5 accounted for 6.58%, and BCS 4 was recorded in only 1.32% of the animals. In contrast, the nervous group showed a more concentrated distribution skewed towards higher BCS values. The most frequent score was BCS 9 (30.8%), followed by BCS 6 and 7 (23.1% each). Only 7.69% of nervous animals had a BCS of 5, and none had a BCS of 4. A general increasing trend was observed in the calm group, with the proportion of animals rising progressively from lower to higher BCS values, peaking at BCS 8. However, this trend declined

slightly at BCS 9, suggesting a partial crescendo pattern. On the other hand, the nervous group displayed a bimodal distribution, with peaks at BCS 6/7 and BCS 9, indicating a less consistent pattern but a strong tendency toward higher BCS values. When comparing the two groups, calm animals exhibited a more evenly distributed BCS pattern, with a marked peak at BCS 8, while nervous animals showed a stronger concentration in the highest categories, particularly BCS 9. These differences might suggest a potential association between temperament and body condition, where nervous individuals tend to have higher BCS, possibly due to stress-related alterations in energy metabolism or feeding behaviour. The overlay of mean ± SEM values further support this pattern.

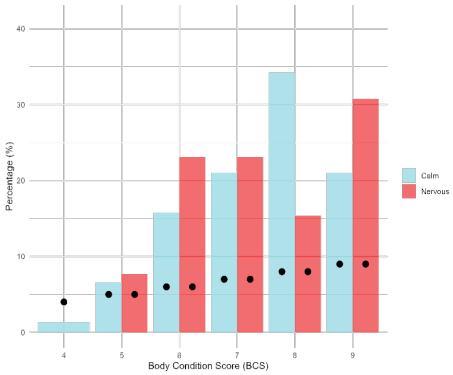


Figure 5. BCS percentage and mean ± SEM for individual score groups in calm and nervous buffaloes

In most BCS categories, nervous animals showed higher mean scores, although with greater variability, especially in the upper BCS range. This was reflected by wider SEM bars, particularly in the nervous group.

The frequency of dirty rumps was observed in 30.3% of the calm group compared to 23.1% of the nervous group, while the frequency of dirty udders was recorded in 44.7% of calm animals and 34.6% of nervous animals. The highest levels of dirtiness conditions were seen in cleanliness of the hind legs, affecting 88.2% of the calm group and 84.6% of the nervous group.

Regarding tarsal joint lesions, these were present in 52.6% of calm animals and 42.3% of nervous animals. Similarly, claw overgrowth was prevalent in both groups, with 78.9% of calm animals and 92.3% of nervous animals being affected (Figure 6). These findings indicate that while both groups face substantial welfare challenges, coat dirtiness conditions were slightly more frequent in the calm group for cleanliness of the rump and cleanliness of the udder, whereas claw overgrowth was more pronounced in the nervous group.

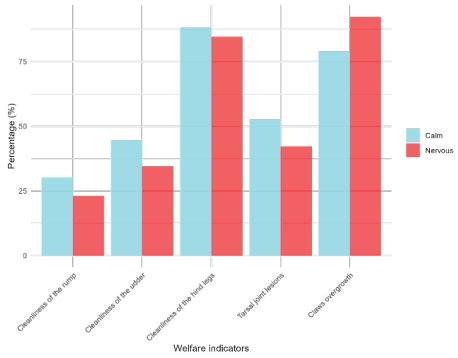


Figure 6. Percentage of poor conditions (score = 2) for welfare indicator in calm and nervous buffaloes

The odds of dirty rumps in the nervous group were 31% lower compared to the calm group (OR = 0.69, 95% CI: 0.23-1.87). However, this difference was not statistically significant (p = 0.485). Animals in the nervous group were slightly less likely to have dirty udders compared to the calm group (OR = 0.65, 95% CI: 0.25-1.62). The difference did not reach statistical significance (p = 0.369). The odds of dirty hind legs were marginally lower in the nervous group compared to the calm group (OR = 0.74, 95% CI: 0.22-2.94), though this result was not statistically significant (p = 0.641). The likelihood of tarsal joint lesions in the nervous group was 34% lower than in the calm group (OR = 0.66, 95% CI: 0.26-1.61). This difference was also not statistically significant (p = 0.365). Animals in the nervous group were 3.2 times more likely to exhibit claws overgrowth compared to the calm group (OR = 3.20, 95% CI: 0.82-21.22). While suggestive of a trend, this result was not statistically significant (p = 0.140, Table 1).

Surprisingly, the present study observed a higher number of animals with soiled body areas among calm buffalo cows compared to their nervous counterparts. The initial working hypothesis assumed that nervous buffaloes

would alternate more frequently between standing and lying positions, leading to increased soiling of the evaluated anatomical regions. A possible explanation for the current findings is that nervous buffaloes, being more excitable, might occupy dominant positions within the social hierarchy, thus gaining preferential access to cleaner resting areas compared to lower-ranking individuals.

Table 1. Logistic regression results comparing welfare indicators between calm and nervous buffaloes (calm group = reference)

Welfare Indicator	Odds Ratio	95% CI Lower	95% CI Upper	p-value
Clean. of rump	0.69	0.23	1.87	0.485
Clean. of udder	0.65	0.25	1.62	0.369
Clean. of hind legs	0.74	0.22	2.94	0.641
Tarsal lesions	0.66	0.26	1.61	0.365
Claws overgrowth	3.20	0.82	21.22	0.140

*CI=confidence interval

CONCLUSIONS

The results of this study suggest that buffalo cows with a calm response during milking exhibit superior performance in terms of milk yield and milking speed compared to their more nervous counterparts.

Although no statistically significant differences were observed in reproductive traits or most welfare indicators, some patterns emerged that may indicate subtle temperament-related effects on body condition and coat cleanliness.

Overall, behavioural responses during milking might serve as a practical and non-invasive indicator for identifying individuals with higher productive potential and could be considered in future management and selection strategies to enhance farm efficiency.

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POLLINATORS IN ROMANIA - ECOLOGICAL AND ECONOMIC CONCERNS

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Abstract

The place and role of pollinators as key elements of the biosphere are well known, as well as their decline and effects at the global level regarding food and economic security. The aim of this paper is to summarize the existing information regarding the species of pollinating invertebrates in Romania, both from an ecological and economic perspective. The information about pollinators comes in unequal proportions from studies carried out in protected and unprotected natural areas, agricultural crops, studies on the effect of some chemicals on the biology and viability of pollinators and from the current legislation. Current information shows that in Romania the situation of pollinators is as alarming as in the rest of the world. Improving the status of pollinators is possible through further scientific efforts, improving the legislation and its application by the book and raising the awareness of civil society, through information, regarding pollinators, starting with the decision makers.

Key words: *common pollinators, ecological and economical interest, invertebrate pollinators, legislation, protected species.*

INTRODUCTION

Usually, speaking about the importance of biodiversity, we refer to the importance of species and habitats in the proper functioning of the biosphere, but, more selfishly somehow, we refer to the benefits they bring to humans. The literature show in numbers, the contribution of pollinating invertebrates to the primary producers existance and to the food security (Ollerton et al., 2011).

The loss/decline of biodiversity, habitats and ecosystem services are effects of anthropogenic activities that began many decades ago.

Alarm signals regarding biodiversity loss led to the adoption of the Rio Convention on Biodiversity (1992) and the signatory states have implemented measures in this regard, with varying degrees of success.

With the increasing population and the increasing need for food sources, a pressing problem has become that of pollinators which are also in decline, beyond their well-known importance in the ecosystem services provided in terms of food security and the perpetuation of biodiversity of primary producers. Given this crisis, Directives 2009/128/EC on using insecticides (Kovacs-Hostyanszk et al., 2016), as well as EU Pollinators Initiative (2019) were adopted.

Romania, as a signatory country to international conventions, is obliged to take pro-pollinator measures, taking into account the level of knowledge at the national level related to both managed and wild species. The international and Romanian scientific community has provided a wide range of information related to pollinators, which has targeted several directions, such as: feeding sources in habitats for pollinators (Cebotari et al., 2017; Sandu et al., 2023; Decourtye et al., 2023), additional sources of feed for hives (Oytun, 2017; Eremia, 2013; Vezeteu et al., 2019), pathogens of bees (Siceanu et al., 2021; Giurgiu et al., 2021; Cebotari & Buzu, 2019; Vezeteu et al., 2019), habitats for pollinators (Sandu et al., 2023; Kovács-Hostyánszk et al., 2016), pollinators and traditional agriculture (Necula et al., 2023; Cebotari & Buzu, 2020; Tăpăloagă et al., 2018; Kovács-Hostyánszk et al., 2016), influence of land management on pollinators (Pătruică et al., 2021; Șonea et al., 2020; Bennett et al., 2018; Demeter et al., 2021), pesticides and pollinators (Aizen et al., 2023), climatic changes and pollinators (Bobiș et al., 2024; Nikolova, 2023; Șurlea et al.,

2023; Nikolova, 2022; Cebotari & Buzu, 2019; Pătruică et al., 2021; Cebotari et al., 2019; Nikolova & Petrova, 2019; Birloiu et al., 2015; Eremia et al., 2015; Decourtye et al., 2013), urban landscape management in relation to pollinators (Süle et al., 2023), role of education and civil society awareness to support of pollinators (Süle et al., 2023; https://wwf.ro/campanii/tobee/, https://www.sor.ro/plante-pentru-polenizatori/, https://mindcraftstories.ro/author/elena-iulia-iorgu/). The above mentioned literature and information sources are only a few of the most recent ones.

This paper focuses only on the species of pollinating invertebrates (*Apis mellifera*, solitary bees, bumblebees, flies - Syrphidae, butterflies and moths, some species of Coleoptera) as the current level of knowledge and their status in Romania, related to the international knowledge.

MATERIALS AND METHODS

This paper presents in a synthetic manner the existing information on pollinators in Romania (invertebrate species) related to international data, both in terms of scientific and socio-economic knowledge.

The information presented comes from scientific publications, but also from data provided by institutions and organizations in Romania, as well as those accessible online for informational and educational purposes.

RESULTS AND DISCUSSIONS

The current state of knowledge of pollinators has shown that the food source and habitats of pollinators and also their diseases are the main elements of concern, as well as the efficiency of supporting tools and measures offered by pro-pollinator legislation and gaps in information from the scientific community.

Information about pollinators in Romania is quite low, disparate and uneven in volume regarding the categories of pollinators (honey-bees, solitary bees, wasps, flies -*Syrphidae*, butterflies and moths, some Coleoptera species) compared to the international one.

The poorest information we have about pollinator species of Coleoptera; the published studies refer to these species but not in terms of their ecological role.

Regarding the specific diversity of pollinators, it can be stated that at present, Romania does not have well synthesized information on pollinator species, be they from the category of managed species or belonging to wildlife. However, we have the Red List of bees in Europe and the IUCN Red List, according to the latter, part of the 630 species of bees are also on the territory of Romania. So, we have concrete information on the status of bee species at European level (Table 1).

Table 1. Status at European level of some groups of pollinating invertebrates (according to WWF Romania via IUCN) (https://wwf.ro/dezvoltare-durabila/)

Status of bees	
Species in decline	37%
Species threatened with extinction	2.45%
Unkonwn status (lack of informations)	57.6%
Status butterflies	
Species threatened with extinction	27%
Status *Syrphidae*	
Species threatened with extinction in the near future	27%

In the European Red List of Hoverflies information from Romania is absent (https://www.iucnredlist.org/regions/europe).

Information from ministerial sources on pollinators in Romania (Ministry of Agriculture and Rural Development, Ministry of Environment, Waters and Forests) is also poor; we find, for example, references on the national action plan on beekeeping, on the development of organic production in Romania and the list of the fertilizers authorised to be used (https://madr.ro/docs/agricultura/fond-funciar/2024/Lista-îngrășăminte-chimice-și-biologice-autorizate-actualizată-la-data-de-1.06.2024.pdf).

On the WWF Romania website instead, we find information about pollinators, especially about the legislation and legislative actions that target them. (https://wwf.ro/noutati/comunicate-de-presa/, https://wwf.ro/resurse/publicatii/).

Before discussing threats to pollinators, which pose challenges to their protection, it is necessary to briefly review the current state of knowledge on the above-mentioned issues.

There are a number of factors that threaten both the diversity of pollinators and the size of

populations and their health. These factors act synergistically and target the most important aspects: food sources and pollinator habitats.

Food sources for pollinators are all species with flowers providing pollen and nectar, including crop species, even weeds. The heterogeneity of the food source plants, the quality of the food (pollen and nectar supply) and the attractiveness of the plants (color and smell - Sandu et al., 2023) are important and determine the wealth of visiting pollinators and the frequency of their visits (Ion & Ion, 2009).

However, there are specific preferences regarding the food source, which explains the presence of certain categories of pollinators on flowering plants. For instance, in greenhouses and crops of tomatoes, strawberries, the most common pollinators are bumblebees while in crops most often honey bees are present.

Also, the species of pollinators visiting the food sources also depend on the species specific dispersal capacity as well as the degree of fragmentation of the landscape in relation to the feeding area; it is understandable why the species of small-sized pollinators (and reduced dispersion capacity) are affected by a large distance between the nesting and feeding sites (Bonmarco et al., 2010; Ricketts et al., 2008; Williams et al., 2010; Winfree et al., 2011).

As for pollinators in urban areas, they find very different food sources here than those in non-urban areas. However, the status of pollinators in cities can be improved by considering pollinator-friendly design of green areas and green spaces, where the pollinators we can meet are *Apidae*, *Andrenidae*, *Syrphidae* (Crișan et al., 2018).

For urban green spaces and gardens, choosing mixtures of less exotic flowering species, and less expensive many times, and preferably with flowering until late autumn, would be helpful for pollinators. It is important to inform civil society about these aspects and overcome negative ideas related to the less managed vegetation and presence of *stinging pollinators* (Süle et al., 2023).

Important for the health of pollinator populations is the access to feeding areas where they find flowering plants by late autumn.

Climate change affects both the food source and the dynamics of pollinator populations. For example, for *Apis mellifera*, high temperatures in autumn and winter cause the early emergence of brood in hives (Șurlea et al., 2023) which leads to the emergence of depleted populations in spring, if an additional food source is not administered to the hives until spring with different food products (Vrabie et al., 2019; Eșanu et al., 2018; Tănăsoiu et al., 2014).

The high temperatures in summer affect pollinators by reducing their feeding period - bees' efficiency varies from species to species as temperature range (Stone, 1994; Kwon & Saeed, 2003).

Indirect effects of high temperatures are due to damage to plants (their scaling) and soil (the nesting site for wildlife suffers from increased ground temperature).

Other climatic phenomena such as snowfall, sleet, hail, frost during the flowering period, affect both the quality and richness of food for pollinators and their mortality.

Additionally, airborne heavy metal pollution is to be monitored as effects on pollen and nectar, given that it has been proven at a national level that the deposition of dust loaded with heavy metals and their accumulation in plant tissues are not to be neglected (Ștefănuț et al., 2018; 2021).

The flowering species polluted by pesticides, herbicides, fungicides and acaricides are both a great and immediate threat to pollinators and in the long run, due to contamination of hives with chemical residues. The immediate threat concerns the diminishing flight capacity and orientation of adults. The indirect threat refers to the depopulation of colonies in time, a phenomenon that is quite difficult to quantify precisely this phenomenon due to the action of other factors, such as diseases, natural mortality, climatic factors (Căuia & Ion, 2023; Martinello et al., 2020; Căuia et al., 2020; Cousin et al., 2019).

The diseases of pollinators are determined by viruses, fungi (*Nosema* genus most frequent), bacteria (*Melissococcus plutonius, g. Spiroplasma*) and *Varroa destructor* (*Acari*) as a transmission agent of the *Vd*MLV virus (Nanetti et al., 2021). For *Varroa destructor* there are numerous studies from which it results that treatments have been designed but even more encouragingly, it has been discovered that certain populations of *Apis*

mellifera develop native, without treatment, resistance to this virus (Cebotari et al., 2019; Vezeteu et al., 2019; Giurgiu et al., 2021; Siceanu et al., 2021).

As far as **habitats** are concerned, all over the world, over the decades, economic, political transformations and military events have occurred, leading to changes in landscape cover and land use, most of which are detrimental to wildlife. Proper habitats for pollinators are grasslands, field crops, gardens, orchards, forests, even hedges, all of them being nesting sites and sometimes, also feeding sites (Sandu et al., 2023), their dimension could be a limiting factor.

It is known that pollinators prefer more natural and semi-natural habitats and less agricultural crops, especially if we are talking about intensive agricultural practices; this is due to the quality of food that habitats provide them and safety as nesting places.

Although most pollinators in crops are honeybees, wild bees present alongside honeybees can sometimes increase the efficiency of pollination (Brittain et al., 2013; Woodcock et al., 2013), especially in crops where weeds are present (e.g. poppies) - are more attractive to pollinators than the "clean", without weeds (Ion et al., 2018).

Negative anthropogenic impact on habitats (degradation, loss, fragmentation, land use change) is also felt by pollinators, who are affected both in terms of specific diversity, especially by specialized species, and in terms of population size (Goulson et al., 2005; Potts et al., 2005; Senapathi et al., 2015; Baude et al., 2016; Sarospataki et al., 2016; Demeter et al., 2021). All these are affecting mostly the above-ground nesting species of pollinators, especially when it comes the fragmentation and loss of habitats (Ferreira et al., 2015; Persson et al., 2015; Redhead et al., 2016; Williams et al., 2010).

Some of these anthropogenic pressures/threats could be diminished by measures such as interspersing of forest strips or patches with flowering species or hedges, to diminish the distances between the nesting patches and feeding ones for pollinators; these corrections come as support for the species with low ability of dispersion (Bonmarco et al., 2010; Ricketts et al., 2008; Williams et al., 2015; Winfree et al., 2011).

Semi-natural grasslands, few remaining in Europe, are ideal habitats for pollinators both as a food source and as a nesting site. However, mowing frequency and the presence of herbivores can alter the quality of this type of habitat.

In Romania, after 1990, with the abolition of cooperatives of agricultural production, and the return of the confiscated lands in the communist period to the former owners/their descendants (Land Restitution Law 1/2000), there have been dramatic socio-economic changes regarding the land use, the use and the market for domestic agricultural products (https://ro.wikipedia.org/wiki/Agricultura_României, https://ro.wikipedia.org/wiki/Apicultura_în_România).

Kremen et al. (2002), Carvell et al. (2006), Harwood & Dolezal (2020) (and not only them) pointed out that the impact of threats varies in intensity from one category of pollinators to another (Winfree et al., 2009, 2011; Quintero et al., 2010; Dore et al., 2021).

The negative impact unanimously felt in the world by pollinators is that of pesticides, whose types and effects are well synthesized by Ara & Haque (2021). Studies on *Apis mellifera* show that pesticide-induced mortality is higher among adults than among juveniles in hives, given that adults come into direct contact with these chemicals, while juveniles only contact with residues brought into hives by adults.

The pesticides used on crops (organochlorines, organophosphates, carbamates, pyrethroids) determine damages on pollinators due to induced effects. The latter category is not widely used because it is not feasible, it is rapidly degraded in the environment (Palmquist et al., 2012). Some pesticides have long-standing environmental resmanence (organochlorines - up to 15 years (according to Jayaraj et al., 2016), are even harmful to humans (e.g. pyrethroids - bioaccumulate in the human body - Dewailly et al., 204), but others have the advantage - that it can be called so - to be degraded by microorganisms (Gangola et al., 2019; Hamada et al., 2015).

This is where European and national legislation aimed at controlling the application of pesticides should intervene. These are Directives 2009/128/EC and the obligation for EU Member States to promote biological methods of pests and reduce the use of insecticides (Kovacs-Hostyanszk et al., 2016), as well as the EU Pollinators Initiative 2019, which refers to wild pollinators.

Member States of the European Union have joined and accepted measures to limit the use of certain categories of pesticides and even ban neonicotinoids as early as 2018.

Romania has appealed six times until 2024 to the Council of Europe for derogation from European legislation on the use of prohibited insecticides (https://wwf.ro/noutati/stiri/).

A decision of the European Court of Justice declares the Romanian derogations from Regulation 1107/2009 to be illegal (wwf.ro/news/).

Why this situation? I think that, first of all, we are talking about insufficient awareness of the role of pollinators in the environment, both at the level of decision-makers and at the level of farmers and civil society in general (the general public).

This is where is felt the need for more information at national level related to pollinators and the risks to which their existence is subject.

Education and information in all ways are welcome, especially in a manner understandable to non-scientists. This is happening, but too little compared to how much is necessary (WWF - bee active, https://mindcraftstories.ro/author/elena-iulia-iorgu/).

Threats to pollinators are both concerns and challenges:

- For scientists - to fill the gaps in the knowledge on pollinators;
- For farmers, land owners and beekeepers - finding ways of compromise between the intensive land use and a pro-pollinators management;
- For decision-makers because it is imperative to review and complement the current legislation in a coherent manner, which also must include also wild pollinators, to follow the exact application of the legislation on the territory of Romania and also to make sustained efforts to support local producers and the opening of markets for indigenous products.

CONCLUSIONS

Given the gaps in the detailed knowledge of pollinator fauna in Romania and the lack of unified databases, further researches are necessary. To improve the situation of pollinators, there is no universally valid method, only directions to follow, meaning concrete actions at the level of threats, with specificities at the national level, depending on the situation.

The necessary changes regarding land use in a pollinator-friendly manner could be possible by promoting the concept of ecological intensification via information, ecological education, completion and correction of current legislation and, last but not least, greater involvement and support of decision-makers related to the problems of beekeepers, land owners and the scientific community.

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FARMERS ATTITUDE TOWARDS COMMON PRACTICES OF BUFFALO CALVES REARING IN ROMANIAN DAIRY FARMING - A SURVEY STUDY

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Abstract

The overall objective of this study was to evaluate the results of farm size influence regarding the management practices from dairy buffalo farms. The current study was based on a survey conducted by online interviews between 2020 and 2021, with a number of 81 dairy farms from Romania. This survey aimed to establish current buffalo calves management technics in Romania along with farmer perceptions surrounding different farm size. The descriptive analysis of farm size influence on calves practices was calculated, with chi-squared tests to assess associations between variables. Concerning the colostrum administration time in buffalo calves, there were significant differences ($p \leq 0.05$) between small farms and large farms, with 0% of the large farms who feed them in first hour of life; and 80% of then small farms who use the same interval. Regarding the calves housing, cow-calf separation and milk quantity offered, we obtained that the size farm it's not a parameter to influence those variables. These results underscored how farm size plays a methodical role in shaping the management strategies employed in rearing dairy buffalo calves.

Key words*: buffalo, calf management, dairy calves, farm size, survey.*

INTRODUCTION

The buffalo (*Bubalus bubalis*) are divided in every continent, they were used for agriculture and dairy industry, being a high adoptable species with less specific food requirements (Silva et al., 2024; Vidu & Bota, 2014). Furthermore, in recent years, buffalo farming has risen to the second place in terms of milk production, with 15% of the world total quantity achieved in 2021. The growing demand for this product is stimulated by its superior nutritional qualities, as well as by the increased public awareness of its benefits (FAOSTAT, 2021; Domenico et al., 2025).

The last update of FAO from 2020 suggests that in Romania has remains 14.000 heads (Minervino et al., 2020). Furthermore, the Association of Buffalo Breeders in Transylvania, registered a little bit more, with a total of 16.721 female, the majority of the owners having traditional farms with a low average of 1.6 head, as a farm size (Jurco et al., 2022). In contrast, in the same decade, Brazil records 1.5 million head of buffaloes, with a specific area, similar with Romania, for the north part of the country (Brazilian Institute of Geography and Statistics, 2021). Within the next few years, overall population of buffalos from Romania, is recomanded to become an important component for improvement to conserving of this genetic stucture (Chiorean et al, 2024).

Accordingly, with all of these aspects, buffalo industry has an important place in agriculture, so that for a successfully production, it is important to manage the animals, with a specific treatment on the youngest calves (Silva et al., 2024).

A growing body of literature has examined the main vulnerable category in dairy farms, calves being conditioned by the intake of colostrum administration in the first days of life for acquirer immunity (Irimia et al., 2021; Lotito et al., 2023) and to survive to stressors during and after calving (Nicolao et al., 2019).

On the other hand, researcher such us Uzturk et al. (2022) underline the existence of critical points in calves rearing practices, starting from calving, colostrum feeding, cow-calf separation and calf housing. Insufficient conditions for calves can increase the morbidity and mortality at this age category, aspect that involve significant negative aspects for the farm economy. However, good management strategies can be improved to decrease the risk of pathogens in buffalo calves (Martinez-Burnes et al., 2024).
Good practices must apply in every farm, although a significant difference in calves management exists between different dimensions of small, medium or large farms (Pempek et al., 2017; Lotito et al., 2023).
Given the lack of knowledge on the effects of farm size on the rearing management of buffalo calves, the aim of the current study was to evaluate the influence of farm size, small (<50 heads) and large (>50 heads), regarding the management practices from Romanian dairy buffalo farms.

MATERIALS AND METHODS

Study population

Approximately 799 dairy water buffalo from eight Romanian farms, were chosen as study population. The buffalo population taken into the study was based on the number of respondents to the questionnaire, more of the farms are located in Transylvania area, distributed during the study period May 2020 - April 2021.
The statistical indicators reflecting the impact of farm size on the technological practices applied to buffalo calves in Romanian dairy farms were calculated using chi square test.
Because the number of respondents was limited, small and medium-sized farms were combined into a single category, named small farms. Thus, the farm size was divided into small farms with 5-50 heads (n = 5 farms) and large farms with over 50 heads (n = 3 farms).

Questionnaire design

An internet and offline questionnaire were considered by using iSondaje.ro platform (iSondaje, 2020).
In total, the questionnaire had 39 questions and was organised in 5 sections as follows: 8, 10, 4, 10 and 13 questions, respectively.
First section of the survey is with a specific focus on herd description with questions designed to capture general farm details, such as milking herd size, cow breed composition of the herd, conventional or organic farming, time for pasture and the geographic position.
Questions from the second section focussed on planning of calving including aspects regarding colostrum and milking, cow-calf separation, were considered and the results were analysed for this paper.
In the third section of the questionnaire gives a brief overview of general calf management, housing and feeding regime.
In Section 4 focus on buffalo calves feeding practices carried out on farm, considering whole milk or replacer, hay type, concentrates protein, food administration and weaning management.
The last section 5 is concerned exclusively on buffalo calves' health and veterinary care.
The questions found in the formulary were open questions, multiple choice, closed and semi-closed questions. The initial testing of the survey was previously performed on a number of 5 farmers from different categories of farms for a good understanding of the questions.

Data analyses

The questionnaire was completed based on telephone interviews, farm visits and online forms; their management being discern by a single interviewer.
All records were individually examined to rule out improbability and the data were recorded in Microsoft Excel 2019 (Microsoft Home & Business English Eurozone). Data were analysed using descriptive statistics applying Chi-square test.
We compare the results to current Romanian and international dairy buffalo industry recommendations.

RESULTS AND DISCUSSIONS

Usage of calving pens, milk type, and cow-calf separation

Data regarding the usage of calving pens, milk type, and cow-calf separation are presented in Table 1.
With a good supply of rearing and health practices in the first three months of buffalo calves, farmers will success in breeding this

species with high performance as adult (Lotito et al., 2023; Silva et al., 2024). Therefore, in small dairy buffalo farms from Romania, calving housing it is practiced in proportion of 80%, while in large farms, regarding pus study, equal proportions of 33.33% are calculate for every type of housing: individual pens, collective pens (Figure 1) and cow-calf contact. Concerning to milk feeding regime, in buffalo dairy farms, all the responds with small farms (100%) and 66.66% of large farms, are using whole milk for the calves.



Figure 1. Buffalo calves housing in collective pens (Own source)

According to the results of the questionnaire, the buffalo cow - calf separation is carried out after 12 or 24 hours postpartum, with 40% for each interval. On the other hand, in large farms (>50 heads), the separation was in proportion of 66.66% abrupt and 33.33% after 12 hours postpartum. These data are in accordance with previous studies where the calf separation from the buffalo mother is carried out 24-48 hours after calving (Mota-Rojas et al., 2019).

The time of first colostrum administration, quantity of first colostrum meal and check the colostrum quality

Table 2 introduce the results concerning the influence of farm size on the time of first colostrum administration, quantity of first colostrum and check the colostrum quality, in buffalo dairy farms.

Accordingly, with the data obtain from the survey, the time for first meal of colostrum has registered only two variables, respectively 1 hour and 1-4 hours *post-partum*, the other variables, 4-6 hours and more then 6 hours were not recorded in the table. However, the timing for the first colostrum meal in the small farms, is carried out in 80% of the time, in the first hour after birth, and the rest of the 20% are manage this in the 1-4 hours interval.

On the other hand, in large buffalo farms, the first colostrum meal is run in the interval 1-4 hours *post-partum*, in a percentage of 100%, according to the questionnaire. This can be based on the fact that in large farms, given the behaviour of buffaloes to withdraw from the herd when calving (De Rosa et al., 2009; Mota-Rojas et al., 2019), they can be identified later through farmers so as to ensure the colostrum of the calves in the shortest possible time. Statistical differences were recorded when comparing small with large farms ($p \leq 0.05$). Furthermore, researchers such as Sturaro et al. (2020), recommend feeding the calves within a maximum interval of 3 hours after calving, with the high-quality colostrum, for superior absorption of immunoglobulins.

In percent of 60% of Romanian farmers, according to the survey, they give an amount 2-4 liters of colostrum to the buffalo calves at the first meal, and the other 40% are giving amounts under 2 liters of colostrum, considering small-sized farms. Regarding the amount of colostrum administered at the first calving, the response option over 4 liters was excluded from the table, because no respondent checked this option.

In large farms, the proportions are 66.66% for the amount 2-4 liters, and 33.33% for amounts under 2 liters. The results obtained are reliable with previous research, where Godden et al. (2019) recommend ingesting an amount of at least 10% and at most 12% of a calf's body weight. In addition, Morin et al. (1997) and Agrawal et al. (2022) found that the most effective immunological capacity for a new born calf is the amount of 4 liters of colostrum for the first meal. Statistical consequences were not recorded for the influence of farm size on the amount of colostrum administered at the first meal.

Table 1. Means (± SEM) CHITEST for usage calving pens, milk type, and cow-calf separation based on farm size

Farm size	Calving pens (%)			Milk type (%)		Cow-calf separation (%)			
	Individual pens	Collective pens.	Cow-calf contact	Whole	Mixt	Abrupt	>12h	>24h	Other
Small farms	0	80	20	100	0	0	40	40	20
Large farms	33.33	33.33	33.33	66.66	33.33	66.66	33.33	0	0
TOTAL	**12.5**	**62.5**	**25**	**87.5**	**12.5**	**25**	**37.5**	**25**	**12.5**
Small vs. Large	NS (0.293)			NS (0.167)		NS (0.160)			

NS p>0.05; * p≤0.05; ** p≤0.01; *** p≤0.001.

According to the questionnaire, in 100% of the dairy buffalo farms in Romania, farmers do not practice checking the quality of colostrum, regardless of the size of the farm of origin. According to previous studies, a high-quality buffalo colostrum has increased concentrations of lactose, fat, vitamin E, A, minerals such as P, Mg, K, Zn, lactoferrin, lysozyme, growth factors, total solids as well as a significant immunological load (Lotito et al., 2023), anti-inflammatory properties (Stoy et al., 2013) antimicrobial or nutraceutical (Ahmadi et al., 2011). In addition, the quality of buffalo colostrum can be analysed using equipment such as a refractometer or colostrometer, the Brix refractometer being the most accessible and handy (Lotito et al., 2023). The statistical results did not show significant differences regarding the influence of farm size on the quality of buffalo colostrum in Romania.

Table 2. Means (± SEM) CHITEST for farm size influence on the time of first colostrum administration, quantity of first colostrum and check the colostrum quality, in buffalo dairy farms

Farm size	First colostrum administration (%)		Quantity of first colostrum meal (%)		Check the colostrum quality (%)	
	0-1h	1-4h	< 2l	2-4l	Yes	No
Small farms	80	20	40	60	0	100
Large farms	0	100%	33.33	66.66	0	100
TOTAL	**50**	**50**	**37.5**	**62.5**	**0**	**100**
Small vs. Large	***(0.028)***		NS (0.850)		NS (1.000)	

NS p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001

Table 3. Means (± SEM) CHITEST for farm size influence on the colostrum administration method, colostrum bank and milk method administration, in dairy buffalo farms

Farm size	Colostrum administration method (%)		Colostrum bank (%)		Milk administration method (%)		
	Bottle	Natural suckling	Yes	No	Bucket	Bottle	Natural feeding
Small farms	0	100	20	80	0	20	80
Large farms	66.66	33.33	33.33	66.66	33.33	66.66	0
TOTAL	**25**	**75**	**25**	**75**	**12,5**	**37.5**	**50**
Small vs. Large	***(0.035)***		NS (0.673)		NS (0.075)		

*NS p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001.*

Colostrum administration method, colostrum bank and milk method administration

Results regarding the method of colostrum administration method, the existence of a colostrum bank and the adoption of the practice of checking the quality of colostrum in dairy buffalo farms, are presented in Table 3.

Regarding the results on the colostrum administration method, the only variable checked by farmers were the bottle/teat and natural sucking from the buffalo cow. The other two options mentioned in the survey, bucket and esophagogastric tube, were eliminated from the table. Moreover, in small-sized farms, natural feeding is an exclusively option to feed the buffalo calves. In contrast, in large farms teat bucket or bottle, was used in a proportion of 66.66%, and natural suckling, is only 33.33% of the interviewed farms. Usualy, the calves feed themselves from the buffalo

dam, without the farmers intervention, as these species are semi-wild animals.
Sometimes buffaloes has different behaviour, especially the primiparous ones, when they refuse the calves (Mota-Rojas et al., 2022), and thus using a teat bottle/buckets or an esophagogastric tube is indicated to administrate the colostrum, which has proven to be the most effective in terms of establishing immunity (Mota-Rojas et al., 2022).
As a practice in dairy buffalo farms, colostrum bank existence, according to the questionnaire 75% of them do not have such a stock, regardless of the size of the farm, large or small. On the other hand, in small-sized farms, 20% of the farmers own a colostrum bank, and in large farms, the percentage rises to 33.33%. A pattern is observed at the level of our country, for the lack of implementation of colostrum banks in farms, because in the case of buffalo farms, this practice has a high percentage of its absence with 66.66% in large farms and 80% in small farms.The need for a colostrum bank is a vital practice for the health of the calves immediately after birth, especially if the buffalo dam has a low amount of colostrum or with a poor quality (Costa et al., 2023).
According to the surveyed farms, milk administration method, were considered three options, such as bucket, teat bottle and natural feeding from the buffalo dam. Automate milk feeder was not an option for Romanian farmers, so it was not included in the processed data.
Thus, in small farms, natural feeding is used in 80% of the interviewed farmers. The adjustment of small farmers to feed calves by natural feeding from the buffalo dam may be due to the stimulation of this species to produce milk in the presence of their own calves, thus the process being easier to manage (Borghese et al., 2007). On the other hand, in large farms, the situation is in contrast, since 33.33% offer milk to the calves in a bucket, 66.66% use a teat, and natural feeding being not an option. The results are similar with Italian farms, where calves are fed with teat bottles in the intensive buffalo breeding system (Borghese et al., 2007). Statistically significant data ($p = 0.075$) were not recorded, however it can be observed a tendancy, in terms of the method of milk administration to calves.

Milk quantity administration, water access and age hay access

Analysed data regarding the amount of milk administered, water and hay access for calves are detailed in Table 4. Parameters such as “under 2 liters” and “8 liters”, regarding the amount of milk administered to calves, were removed from the table, because no farmer checked these options.

Table 4. Means (± SEM) CHITEST for farm size influence on the milk quantity administration, water access and on the age hay access, in buffalo dairy farms

Farm size	Milk quantity administration (%)			Water access (%)	Age hay access (%)		
	Ad libitum	4 l/day	6 l/day	Ad libitum	1-3 weeks	4-8 weeks	> 8 weeks.
Small farms	80	20	0	100	40	60	0
Large farms	0	66.66	33.33	100	0	66.66	33.33
TOTAL	**50**	**37.5**	**12.5**	**100**	**25**	**62.5**	**12.5**
Small vs. Large	NS (0.075)			NS (1.000)	NS (236)		

NS $p>0.05$; *$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$.

In addition, 80% of small farms, regarding the survey generated data, offered *ad libitum* access to milk, and 20% the amount of 4 liters/day. On the other hand, in large farms, the percentage is different, calves are fed 4 liters of milk per day in a percentage of 66.66%, while 33.33% of respondents feed them with 6 liters/day. These values are in accordance with previous studies, where the proportion of milk recommended to be ingested by calves, regardless of species, is within the range of 10-12% of the animals weight (Godden et al., 2019). No significant differences were recorded, however we can observe that there is a trend regarding the influence of farm size on the amount of milk administered to buffalo calves.

The farmers responses were unanimously in favor of ad libitum water access for 0-3 months calves aged, the other options of 2 liters/day and 5 liters/day not being taken into account, and thus were not included in the table. The results are in agreement with previous research in which water intake at a young age is recommended for rumen development and feed digestion (Macovski, 2018; Dairy-cattle, 2019). In addition, Council Directive 2008/119/EC of 18th of Decembre 2008 specifies the importance of water, especially after the age of two weeks and especially during droughty periods.

According to the survey data, hay access in buffalo calves in small-sized farms is it starting aroung 4-8 weeks of life, for 60% of the farms, and for the rest of 40%, this feeding regime is it starting earlier, from 1-3 week after birth. Furthermore, hay access in large farms is 66.66% from 4-8 weeks of live and 33.33% at ages over then 8 weeks of life. Previous research has shown that hay access at young ages brings calf digestive development in the same way as feeding with starter feeds (Francia et al., 2009). Studies on this variable are limited, so that statements can be strictly in the form of an supposition. Statistical data have not been recorded regarding the age of hay access to buffalo calves.

CONCLUSIONS

According to the results obtained, the moment and the method of colostrum administration in water buffalo calves are factors that can are influenced by the size of the farm. However, colostrum management in the first period of life, in calves, can promote better productive management of buffalo calves in the face of possible health issues.

In addition, practices regarding milk feeding, housing, colostrum bank, cow-calf separation, water and hay access, cannot be considered to be influenced by such a factor studied.

Considering the results obtained in this study, the success for a health and strong adult buffalo, it's crucial to respect and understanding the management of calves in first three months of life. Moreover, significant differences could be determined between small and large sized of Romanian buffalo farms.

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FACTORS INFLUENCING THE QUALITY OF TURKEY MEAT

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Abstract

Genetic, technological, nutritional, and breeding factors influence the quality of turkey meat. In terms of its biochemical composition, turkey meat is characterized by a high protein content and a favourable ratio between unsaturated and saturated fatty acids, which makes it a valuable nutritional source. Also, turkey meat is lower in fat compared to other types of meat. Regarding the quality of turkey meat, it varies depending on the anatomical part used (breast and thighs) and has distinct nutritional values. Processing factors, storage temperature, and post-mortem pH of the meat affect sensory properties such as texture, taste, and colour. Improving the quality of turkey meat involves optimizing growing conditions, as well as correctly managing the slaughtering and preservation processes to prevent damage to organoleptic characteristics and food safety.

Key words*: quality meat, turkey, antioxidants.*

INTRODUCTION

Appreciated for its high nutritional value, low fat content, and balanced protein profile, turkey meat is favored by consumers in many parts of the world (Bianchi & Fletcher, 2002; Baracho et al., 2020; Moise et al., 2024).

On the other hand, it is well known that the qualitative parameters of this type of meat can be influenced by a series of biological and environmental factors (genetics, sex, feeding regime, rearing system, pre-slaughter stress, processing conditions, etc.) (Debut et al., 2003; Alvarado & McKee, 2007; Pietrzak et al., 2013), which also negatively affect its stability during processing and storage (Deeb et al., 1999; Leishman et al., 2022).

For example, genotype and sex influence the texture and color of the meat, as well as the protein and fat content (Cizek, 2019; Costache et al., 2019; Tudorache et al., 2022), while growth and feeding methods affect the development rate and chemical composition of the tissue, influencing the tenderness and juiciness of the final product (Custură et al., 2012; Tougan et al., 2013; Zhang et al., 2015; Pulido & Toledo, 2021). Additionally, environmental factors such as temperature, humidity, and light play a crucial role in the development of muscle mass and the maintenance of bird health (Ferrante et al., 2013; Geers & Madec, 2015; Chang et al., 2021); studies in this regard indicate that a stressful or inadequate environment can negatively affect muscle metabolism, causing undesirable changes in pH and water retention, negatively impacting meat quality (Marchewka et al., 2013; Fraqueza, 2015).

Another essential aspect in determining quality is the influence of factors acting during the slaughter of birds, such as post-mortem processing and handling conditions, storage temperature, etc. (Owens et al., 2009; Okuskhanova et al., 2017); improper transport and handling of birds before slaughter contribute to the onset of stress, resulting in inferior meat quality, characterized by defects such as pale, soft, and exudative meat (PSE - Pale, Soft, Exudative), similar to that found in other animal species (Fletcher, 1995; Woelfel et al., 2002; Owens et al., 2009; Bilgili et al., 2019).

In this context, the present paper aims to analyze in detail the factors that influence the productive performance and meat quality of turkey broilers, to provide a general perspective on the possibilities for optimizing the growth and processing of this category of birds, with a view to obtaining a high-quality final product.

MATERIALS AND METHODS

For the completion of this work, scientific articles, specialized books, and official reports published between 2000 and 2023 were consulted, available in databases such as Google Scholar, Scopus, and on the websites of recognized institutions (EFSA, USDA, ISO). The key words used in the search included: "turkey meat quality", "poultry meat", "PSE meat", "processing methods for turkey meat", "nutrition and turkey growth", and "poultry meat safety".

The selection of works was based on their relevance to the proposed topic, considering both experimental studies and review articles. Only materials that directly analyzed the factors influencing turkey meat quality (genetic, environmental, nutritional, and technological factors), as well as aspects related to food safety, were included. The extracted information was then grouped and synthesized according to the categories of factors presented in the text, with the aim of providing a clear and accessible overview of the main conclusions from the specialized literature.

RESULS AND DISCUSSIONS

1. Biological factors that influence the quality of turkey meat

The quality of turkey meat is determined by a series of essential biological factors, such as genetics, sex, age, and the physiological condition of the birds (Leishman et al., 2022; Adams & Singh, 2023). These factors influence the sensory and physicochemical properties of the meat, such as texture, color, tenderness, juiciness, and water-holding capacity (Petracci & Cavani, 2012; Sarantopoulos et al., 2016).

1.1. *The genotype and its impact on meat quality*

The physical and chemical properties of turkey meat are mostly determined by genetics (Deeb et al., 1999; Pietrzak et al., 2013; Marangoni et al., 2015). Turkeys from different genetic lines differ significantly in terms of meat quality, muscle size, and tissue composition (Lonergan et al., 2003; Cizek, 2019).

A higher proportion of muscle tissue is found in genetically selected turkey lines for rapid growth, but there is also a higher chance of muscular anomalies such pale, soft, and exudative (PSE) meat (Owens et al., 2009; Pietrzak et al., 2013). This results from modifications in muscle metabolism, which can cause reduced water retention and a low post-mortem pH, which reduces the end product's appeal to buyers (Castellini et al., 2002; Lu et al., 2020). However, the muscle yield is lower in traditional turkey breeds with slower growth rates, but the meat is tougher and more juicy (Serbest, 2015; Hiscock et al., 2022). Therefore, genetic selection must be carefully used to manage the trade-off between intensive production and meat quality (Northcutt, 2001; Adams & Singh, 2023).

1.2. *The impact of sex on the characteristics of meat*

Bird sex has a major impact on meat sensory qualities, tissue distribution, and body composition (Tougan et al., 2013; Schantz et al., 2019). According to studies, male turkeys gain more muscle mass than females, which makes their meat thicker but less soft (Zhang et al., 2018; Hiscock et al., 2022).

Women, on the other hand, have a greater percentage of intramuscular fat, which improves the meat's flavour and juiciness (Berri, 2008; Ferrante et al., 2013). Additionally, studies reveal that females have more constant post-mortem pH, which contributes to the meat's better palatable appearance and consistent colour (Debut et al., 2003; Avila & Alvarado, 2013).

Due to consumer preferences for the more tender and juicy meat, these sex variations are significant in the turkey meat industry and can affect the choice of birds for processing (Fletcher, 2002; Berri, 2008).

1.3. *The age of birds and its effects on meat quality*

The meat's texture, colour, and juiciness are all directly impacted by the age at slaughter (Miao et al., 2019; Berri et al., 2020). The customer experience may suffer as older turkeys acquire more fibrous muscle tissue, while younger turkeys often have more soft and juicier meat (Lyon et al., 2004; King & Whyte, 2006).

Studies have shown that young turkeys contain more soluble collagen, which makes their meat

softer and more palatable to chew (Owens et al., 2009; El-Bahy et al., 2021). On the other hand, older birds' greater muscle mass and rest cause insoluble collagen to build up, making their meat stiffer and more challenging to prepare (Fletcher, 1995; Sarantopoulos et al., 2016). Age affects the meat's colour in addition to its texture. Due to pigment buildup and modifications in haemoglobin metabolism, older turkeys have darker meat (Geers & Madec, 2015; Leishman et al., 2022). Given that colour is a key signal of the product's freshness and quality, this change may affect how consumers perceive the product.

1.4. *Physiological state and stress before slaughter*

The amount of stress that birds endure prior to slaughter is a crucial factor that affects the quality of their meat (Marchewka et al., 2013; Awan & Rahman, 2020). Stressor exposure can cause metabolic alterations that impact muscle pH and water retention, including incorrect handling, transportation, and unsuitable environmental conditions (Heckendorn et al., 2015; Bilgili et al., 2019). Stress raises cortisol levels and triggers anaerobic metabolism, which can result in meat that is PSE (pale, soft, exudative) and a sharp drop in post-mortem pH (Marchewka et al., 2013; Avila & Alvarado, 2013). This phenomenon affects consumer acceptance by compromising the meat's texture and look (Owens et al., 2009; EFSA, 2020).

On the other hand, meat from birds grown in ideal conditions with appropriate stress management has a balanced pH and improved sensory qualities (Ferrante et al., 2013; Pulido & Toledo, 2021). To increase the quality of the finished product, the turkey meat business employs techniques like managing the birds carefully, employing suitable transportation circumstances, and lowering pre-slaughter stress (ISO 22000:2018; USDA, 2021).

2. Environmental and nutritional factors that influence the quality of turkey meat

In addition to biological characteristics, environmental and nutritional conditions can affect the quality of turkey meat (Woelfel et al., 2002; Ferrante et al., 2013). The development of the birds and the final properties of the meat are greatly influenced by the growing environment, temperature, humidity, illumination, and feed composition (Chang et al., 2021; Leishman et al., 2022). The final product's texture, colour, softness, and stability can all be enhanced by properly managing these variables (Sante & Voilley, 2021; Chen et al., 2023).

2.1. *Ambient temperature and humidity*

Bird health and metabolism are directly impacted by environmental factors including temperature and humidity (Ferrante et al., 2013; Tougan et al., 2013; Pulido & Toledo, 2021). Meat quality can be adversely affected by extreme temperatures since they can cause stress and interfere with muscle development (Alvarado & McKee, 2007; Petracci & Cavani, 2012).

Temperatures above 30°C raise the danger of heat stress, which reduces food intake and, indirectly, inhibits the formation of muscle mass (Owens et al., 2009; Fraqueza, 2015). This condition may result in less juicy and tender meat. Meat at low temperatures (less than 10°C) uses a lot of energy for thermoregulation, which might affect tissue distribution and result in tougher, more collagen-rich meat (Marchewka et al., 2013; Lu et al., 2020).

Furthermore, birds that experience high humidity levels (>70%) may develop respiratory issues, which may hinder their growth and indirectly impact the quality of their meat (Bianchi & Fletcher, 2002; Adams & Singh, 2023). Conversely, low humidity (<40%) might cause muscular tissues to become dehydrated, which will reduce the meat's softness (Cizek, 2019).

Turkeys develop healthily and produce high-quality meat when kept in a controlled environment with an ideal temperature of 18–22°C and a relative humidity of 50-60% (Avila & Alvarado, 2013; Arslan & Seker, 2021).

2.2. *Breeding systems and bird density*

Turkeys' wellbeing, health, and muscle growth are influenced by the density at which they are grown (Leishman et al., 2022; Chen et al., 2023).

Birds with high densities (>40 kg/m^2) have less room to move, which can result in inadequate muscular growth, poor muscle oxygenation, and excessive fat buildup (Hiscock et al., 2022; Bolek & Węglarz, 2022).

Low densities (less than 30 kg/m^2) enable more vigorous movement, which enhances muscle growth and the meat's ideal protein content, but they also raise production costs (Johnson, 2020).

To get beef with the ideal ratio of tenderness to firmness, density and comfort must be balanced (Marchewka et al., 2013). Furthermore, meat from birds maintained in large systems with outdoor access has greater intramuscular fat, which improves the meat's flavour (Castellini et al., 2002; Leishman et al., 2022).

2.3. *Illumination and its influence on muscle metabolism*

Turkeys' feeding habits, metabolism, and muscle growth are all impacted by lighting (Sarantopoulos et al., 2016; Awan & Rahman, 2020). Research indicates that the kind and length of light can have an impact on the meat's softness and muscle fibre structure (Marchewka et al., 2013; El-Bahy et al., 2021).

Meat that is exposed to constant lighting (24-hour light) may become tougher and less juicy due to stress and hyperactivity (Okuskhanova et al., 2017; Hiscock et al., 2022).

A higher protein content in meat, balanced growth, and ideal muscle mass development are all facilitated by intermittent lighting (16 hours of light and 8 hours of dark) (King & Whyte, 2006; Bayraktar et al., 2021).

Birds' physiological responses are enhanced by natural or selectively spectrum lighting (blue/green LEDs), which results in more succulent and juicy meat (Lonergan et al., 2003; Correa et al., 2019).

The quality of the finished product can therefore be greatly impacted by appropriate lighting management in turkey farms (Hiscock et al., 2022).

2.4. *Nutrition and food composition*

Nutrition is one of the most important factors influencing the development of muscle tissues, meat color, and protein stability (Qiao et al., 2002; Marangoni et al., 2015; Barroeta, 2018).

Proteins and amino acids. Turkeys need a balanced intake of proteins and essential amino acids for optimal muscle development (Castellini et al., 2008; Said et al., 2022). High-quality proteins (e.g., soy, peas, fish meal) contribute to more tender and juicier meat with a high protein content (Berri, 2008; Guo et al., 2018). Protein deficiency leads to stunted growth and less dense, softer meat (Debut et al., 2003; Alvarado & McKee, 2007).

Fats and fatty acids. Omega-3 and Omega-6 fatty acids influence the consistency and stability of fats in meat (Zhang et al., 2018; Sante & Voilley, 2021). Omega-3 enriched diets can improve the lipid profile and flavor of meat (Samuel et al., 2022). Excess saturated fats in the diet can lead to greasier meat with lower sensory quality (Correa et al., 2019).

Vitamins and minerals. Vitamin E and selenium protect muscle cells against oxidative stress, preventing meat rancidity and loss of natural color (EFSA, 2020; Hiscock et al., 2022). Zinc and copper deficiency can reduce the water-holding capacity of meat, affecting its texture and juiciness (Said et al., 2022).

A balanced diet, based on the optimal ratio of proteins, fats, and micronutrients, is crucial for obtaining high-quality meat (ISO 22000:2018; Del Valle et al., 2023; Custură et al., 2024).

3. Technological factors that influence the quality of turkey meat

The stability, texture, juiciness, and safety of the finished product are all greatly impacted by the processing and storage of turkey meat (Hudson, 2012; Awan & Rahman, 2020; Chen et al., 2023). The following are some of the most significant technological elements influencing turkey meat quality:

- techniques for post-mortem handling and slaughter;
- meat storage and cooling;
- the impact of thermal processing on texturing;
- preserving quality through packaging and preservation.

We will examine each of these elements and how they affect the end product's quality in this section.

3.1. *Techniques for post-mortem sacrifice and manipulation*

Muscle pH, texture, and water retention are influenced by the way turkeys are killed and treated after slaughter (Table 1) (Fletcher, 2002; Alvarado & McKee, 2007; Owens et al., 2009). Protein stability and the rate of pH drop are impacted by slaughter practices. Defects

like dry and firm meat (DFD - Dark, Firm, Dry) or pale, soft, and exudative meat (PSE) could arise from improper slaughter (Knox et al., 2012; Mendes, 2017).

Table 1. The influence of the slaughter method

Method of sacrifice	Impact on meat quality
Slaughter by electric stunning	High stress risk results in PSE meat
Sacrifice by gas (CO_2)	Reducing stress results in more tender meat
Slaughter Halal/Kosher (without stunning)	Risk of rapid pH drop results in drier meat

Research indicates that CO_2 gas stunning is associated with superior meat quality, as it reduces bird stress and slows down post-mortem pH decline (Owens et al., 2009; Wilkins, 2015).

3.2. *Cooling and storing meat*

After slaughter, cooling is essential to prevent bacterial growth and maintain freshness (Barroeta, 2018; USDA, 2021). Rapid cooling helps stabilize the pH and reduce the risk of developing PSE and DFD defects (Table 2) (Geers & Madec, 2015).

Table 2. The impact of cooling methods on meat quality

Cooling method	Time	Impact on quality	Details
Cooling in the air (2-4°C)	4-6 h	Reduced moisture loss, tender meat	Air cooling is the most commonly used method in Europe because it preserves the natural texture and color of the meat
Cooling in the whater (0-2°C)	2-4 h	Increased water retention, but risk of contamination	Cooling in water is faster, but it can lead to excessive water retention, affecting the final texture
Cryogenic cooling (-40°C)	<1 h	Rapid crystal formation → affects texture	Faster freezing is used for export, but it can cause the formation of large ice crystals, which degrade the structure of muscle fibers.

3.3. *Thermal processing and its impact on texture*

Turkey meat is often subjected to thermal processing (boiling, roasting, frying, smoking) (Hudson, 2012; Awan & Rahman, 2020). High temperatures can affect protein stability and water retention, leading to changes in texture (Table 3) (El-Bahy et al., 2021).

Table 3. The impact of thermal processing methods on meat quality

Processing type	Temperature (°C)	Impact on meat	Details
Fever	70-80°C	Soft texture, loss of nutrients	Boiling reduces water retention capacity, which can affect juiciness
Baking	150-180°C	Good juice retention, crispy crust	Baking at moderate temperatures maintains the tender texture and reduces water loss
Frying	>180°C	Water loss, firmer meat	Frying at very high temperatures can make the meat drier and tougher, affecting its quality
Smoking	40-60°C	Improved taste, antimicrobial effect	Smoking enhances the taste and shelf life, but it can alter the structure of proteins

To maintain quality, methods such as baking at moderate temperatures or sous-vide techniques (slow cooking at low temperatures) are recommended, which can help preserve the tenderness of the meat (Fletcher, 2002; Castellini et al., 2008).

3.4. *Packaging and preserving turkey meat*

Packaging is essential for maintaining freshness and preventing oxidation (Table 4) (Wilkins, 2015; Hiscock et al., 2022).

Table 4. Packaging and preserving turkey meat

Type of packaging	Advantages	Disadvantages	Details
Vid (vacuum)	Prevents oxidation, maintains freshness	High cost	Vacuum packaging is the most effective method for preserving the quality of meat, preventing dehydration and oxidation
Packaging in a modified atmosphere (MAP - $CO_2/N_2/O_2$)	Increases lifespan, prevents bacterial growth	It requires special equipment.	MAP (modified atmosphere packaging) maintains color and freshness, but requires precise gas control
Traditional packaging (plastic, paper)	Reduced cost	Short storage period	Traditional packaging is the most commonly used for quick sales, but it offers the shortest shelf life

For export and long-term storage, vacuum packaging and quick freezing are the most effective methods (Alonso-Calleja et al., 2004; ISO 22000:2018).

4. Food safety and consumer requirements regarding turkey meat

In addition to biological, environmental, and technological aspects, food safety is an essential factor in maintaining the quality and acceptability of turkey meat (Heckendorn et al., 2015; EFSA, 2020). Food safety regulations aim to control bacterial contamination, the use of additives, and processing standards, ensuring that the final product is safe for consumption (ISO 22000:2018; USDA, 2021).

At the same time, consumer preferences are influencing the turkey meat industry, driving demand for healthier, more natural products with high sensory quality (Berri, 2008; Hiscock et al., 2022).

4.1. *Microbiological contamination and safety measures*

Salmonella spp., *Campylobacter* spp., *Listeria monocytogenes* and *Escherichia coli* are among the bacteria that can contaminate turkey meat (EFSA, 2020; Samuel et al., 2022). If the product is not handled and processed correctly, these bacteria can result in serious food poisoning (Owens et al., 2009; Fraqueza, 2015).

Contamination prevention measures. Equipment should be cleaned and disinfected in accordance with good hygiene practices (GHP) (ISO 22000:2018; Correa et al., 2019).

Keeping an eye on storage temperatures: cooked meat should be kept at 60°C and raw meat below 4°C (Fletcher, 2002; USDA, 2021).

To control microbiological hazards, the HACCP approach is applied, which identifies important points in the manufacturing chain (Owens et al., 2009; EFSA, 2020).

Using films containing antimicrobial compounds to prevent the growth of diseases, also known as antibacterial packaging (Marangoni et al., 2015; Awan & Rahman, 2020).

4.2. *Food additives and preservatives used in processed meat*

Food additives and preservatives may be added to processed turkey meat to preserve freshness and stop bacterial growth (Table 5). Enhancing the flavour, texture, and shelf life is their responsibility (Hudson, 2012; Jensen & Dolberg, 2021; Hiscock et al., 2022).

Table 5. Food additives and preservatives used in processed meat

Aditives	Role	Possible negative effects
Nitrites and nitrates	Prevent the growth of bacteria, stabilize the color	Risk of nitrosamine formation (potentially carcinogenic)
Salt and phosphates	They improve water retention and texture	Excessive consumption can affect cardiovascular health
Antioxidants (e.g., ascorbic acid, tocopherols)	Prevent the rancidity of fats	Safe within the recommended limits
Natural extracts (rosemary, green tea)	Natural preservatives, enhance the flavor	They have no known side effects

The current trend is to reduce the use of synthetic additives and replace artificial preservatives with natural extracts, responding to consumer demands for healthier and less processed products (Bianchi & Fletcher, 2002; Okuskhanova et al., 2017).

4.3. *Consumer perception of turkey meat quality*

Modern consumers are increasingly concerned about the origin, safety, and composition of the meat they consume (Northcutt, 2001; Marchewka et al., 2013). Market studies show that their preferences are influenced by appearance, texture, taste, and nutritional information (Table 6) (Miao et al., 2019; Bolek & Węglarz, 2022).

Table 6. Consumer perception of turkey meat quality

Selection criterion	Influential factors	Impact on the decision
The color of the meat	pH, refrigeration methods	Consumers prefer uniform pink color, avoid pale or dark meat
Tenderness and juiciness	Species, maturity, processing	The soft and juicy texture is preferred, avoiding dry meat.
Origin and cultivation method	Organic farms vs. intensive farming	Products labeled "eco" are more appreciated, even if they are more expensive
The presence of additives	Preservatives, colorants, nitrites	Consumers prefer meat without synthetic additives

Current trends show an increase in demand for organic meat, free from antibiotics and artificial additives. In addition, many consumers choose meat from ethical farming systems, with animal welfare respected (Berri et al., 2020; Arslan & Seker, 2021).

4.4. *Labeling and quality standards*

Products made from turkey meat must be properly labelled to enable customers to make educated decisions (ISO 22000:2018; Jensen & Dolberg, 2021). Labels are required by FDA and EU laws to provide unambiguous information regarding:

Origin and method of production (e.g., "organic", "raised without antibiotics")

Values of nutrition (calories, proteins, fats, and vitamins)

Date of expiration and method of preservation

Ingredients and potential allergies listed

The European Food Safety Authority's (EFSA) rules, which set stringent limits on antibiotic residues and pollutants, govern quality standards in the EU (EFSA, 2020). Consumer confidence and product safety are guaranteed using these standards (Awan & Rahman, 2021; USDA, 2021).

CONCLUSIONS

From the study of specialized literature regarding the factors that influence meat production in turkey broilers, it has been concluded that genetic type and sex directly influence the texture and tenderness of the meat, while slaughter age and rearing conditions determine some aspects related to the color and juiciness of the meat.

Regarding environmental factors, it has been noted that temperature and humidity play a major role in the development of muscle masses, while controlled lighting programs can influence metabolism and, implicitly, meat quality.

Nutrition is another determining element, as the turkey broiler requires a balanced diet rich in proteins, essential fatty acids, and micronutrients to obtain meat with optimal nutritional value and superior sensory characteristics.

Processing live birds was another subject of our study, concluding that transport stress, stunning method, scalding regime, carcass cooling system, and packaging technique influence both the quality and shelf life of the obtained meat.

Another important element of interest was food safety, as preventing microbiological contamination is an essential objective in the meat industry; the use of strict hygiene measures.

Additionally, the increased demand for healthy and natural products has prompted the industry to reduce the use of synthetic additives and place greater emphasis on clear labeling and quality certifications.

Looking to the future, it becomes increasingly evident that sustainability will play a central role in the development of the sector, with the main directions in this regard being the

reduction of environmental impact, the use of alternative food sources, and the improvement of product traceability; additionally, the adoption of new processing and preservation technologies will contribute to increasing product sustainability and offering safer and healthier options for consumers.

From the presented information, the final conclusion is drawn that the quality of turkey meat is the result of a complex interaction between biological and environmental factors, and understanding these allows for the improvement of the sensory and nutritional characteristics of the final product, while also providing the possibility to adapt production to the increasingly demanding requirements of consumers.

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LIVESTOCK OWNERS` ROLE IN ANIMAL AND FARM REGISTRATION THROUGH NEW ELECTRONIC FUNCTIONALITIES

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Abstract

Current strategies for control of contagious animal diseases rely on traceability of all animal movements. For this purpose, the identification and registration (I & R) of animals and animal holdings is of crucial importance. In Bulgaria, all data on animal I&R are maintained by the integrated information system VetIS, operated by the national competent authority Bulgarian Food Safety Agency. For the improvement of the system new functionalities have been developed, giving active access to farmers. Through an anonymous survey we investigated livestock owners` perceptions on their new role in VetIS. The results showed that one-third of the respondents believed their active access will raise their compliance with the legislative requirements through facilitating the paper work on the farm and avoiding error entries on the animal status related to birth, movement, slaughter or death.

Key words*: animal registration, electronic identification, farmers, livestock, VetIS.*

INTRODUCTION

The management of information on the control of communicable diseases and food safety is directly linked to the identification of farm animals for the purpose of traceability (Zhao et al., 2020) and the consumers` requirements for health guarantees regarding products of animal origin (Mascarello et al., 2024; Zimmermann et al., 2024). The effective use of this information can be ensured through a specialized database on all animal movements with regard to animal health events and trade (Meisinger et al., 2008; Murphy et al., 2009), which in Bulgaria was developed in 2010 and maintained by the integrated information system of the Bulgarian Food Safety Agency (BFSA) – VetIS. Similar type of digitalized records on data on animals and farms are used in many developed countries with clear policies on food safety and management of animal health like the European Union, the USA, Canada, Australia (Bass et al., 2008; Bowling et al. 2008; CRS, 2010; Vlad et al., 2012).

As the responsibilities for managing the animal identification and traceability systems are in general within the national regulatory bodies, the participation of the farmers in the process is based on a mandatory principle under certain legal requirements (CRS, 2010; EU, 2015; EU, 2016; EU, 2019; EU, 2021). Due to the clear understanding that the entered data will be most up-to-date if the responsibility for their entry and control over the updating process of the data is assigned to the operator of the farm (Huang & Fu, 2023a), a new development of the VetIS functionality started in Bulgaria with several open to the authorized stakeholders' options.

The information system ensures effective means for searching and providing each user with data and documents about his own farm holdings and animals registered in them, similarly to other applications already functioning in some EU member states (DAFM, 2022).

The defined rules and requirements for the type, volume of data to be entered and used are in accordance with the Bulgarian Law on Veterinary Activities (National Assembly, 2006) and the synchronized European legislation on animal identification and registration.

Gaining understanding on the factors that increase the usefulness of the system for the farmers, would be a valuable tool for the policy-makers in increasing the effectiveness of the VetIS information system, respectively the benefits for animal health control and food safety management.

MATERIALS AND METHODS

Study model

The authors prepared and disseminated personally an anonymous written questionnaire

with closed-ended questions among farm animal owners from the settlements in the Yambol region in the period February-March of 2024. All the respondents were initially informed about the voluntary character of the survey and the possibility to withdraw at every stage, with full protection of their personal data. Every farmer willing to participate was given a paper questionnaire with multiple-choice questions to fill in anonymously. All completed questionnaires were returned to the authors (n = 138), representing heterogeneous respondents from the whole area. This study did not need an approval of an ethics committee, as no manipulation or interventions on humans were conducted.

With the new functionalities being developed in addition to the VetIS, it is planned that every owner will have the right to access the data in the system, which are necessary for the fulfilment of his responsibilities, and the access to the system will be consistent with his rights to add, edit and use (see) data from it, according to the nature of the activity. The system will allow the owner to create (enter, edit and view) in Bulgarian, certain data related to the livestock holding of which he is the owner or tenant and the animals that reside on his property.

The questions were divided into several sections. The first section (questions 1-5) contained questions on the respondents` demographic characteristics, including their age, gender, education, species and number of productive animals kept. The second section (questions 6-12) focused on the awareness and attitudes of the respondents about the work with the integrated information system of the BFSA - VetIS and the envisaged new functionalities with active access for farmers: increasing the relevance of the data in the system; reduction of violations within the deadlines for animal identification; facilitating and increasing the percentage of submitted notifications for death/slaughter of animals; improved farm management and simplified documentary work; reducing the discrepancies between the animals entered in the system and their actual number; possibility to control and correct the discrepancies in VetIS regarding the animals in the establishment.

The completed questionnaires were returned to the authors and subsequently coded with numerical values, as each text answer was converted to a number for easier data analysis.

Statystical analysis

The obtained data were subjected to statistical processing (IBM SPSS-Inc., 2019, SPSS Reference Guide 26 SPSS, Chicago, USA). The studied parameters were analyzed using descriptive statistics (frequency distribution), correlation (Pearson coefficient) and chi-square. Results were considered significant at p values <0.05 (two-tailed). The obtained results were then presented in tables and diagrams (Excel, Windows 10).

RESULTS AND DISCUSSIONS

The respondents in our study represented various groups distributed by age, gender and education (Table 1).

Table 1. Farm animal owners` distribution by their demographics

Respondents` characteristics	Number	Percentage
Gender */Mean 1.79; Std. Dev. ± 0.41/*		
1) Female	28	20.29
2) Male	108	78.26
Age (years) */Mean 2.46; Std. Dev. ± 0.85/*		
1) 20-29	4	2.89
2) 30-60	98	68.12
3) 61-64	11	7.97
4) 65+	28	20.28
Education */Mean 3.01; Std. Dev. ± 0.82/*		
1) Primary school	5	3.62
2) Middle school	18	13.04
3) High school	96	69.57
4) Bachelor degree	5	3.62
5) Master degree	12	8.70
Farm animal species */Mean 1.83; Std. Dev. ± 1.20/*		
1) Cattle	68	49.28
2) Sheep	49	35.51
3) Goats	3	2.17
4) Several animal species	14	10.14
Number of farmed animals */Mean 3.32; Std. Dev. ± 1.22/*		
1) from 1 to 9	4	2.90
2) from 10 to 49	38	27.54
3) from 50 to 99	35	25.36
4) from 100 to 149	31	22.46
5) from 150 to 499	23	16.67
6) 500 and more	4	2.90

*Some values do not represent 100% due to non-respondents.

Most of the participants in the survey were men (78.26%), the majority of respondents were between 30 and 60 years old (68.12%), high school graduates (69.57%). These parameters were comparable for the Southeastern economic

region of Bulgaria, within which Yambol is included, as official data reported predominant share of 60.7% men, representing the labour force there (NSI, 2023). Almost half of the farmers raised cattle (49.28%), just over a third raised sheep (35.51%), and only 2.17% of farmers raised goats. Some respondents (10.14%) stated that they kept several animal species at the same time, usually sheep and goats. Data on the number of animals in livestock farms showed that the study involved owners of smaller farms with 10 to 49 animals (27.54%), medium-sized farms with 50-99 animals (25.36 %) and larger farms from 100 to 149 animals (22.46%) which data corresponded with the structure of the animal husbandry in the region and the tendency of increasing the number of animals on the farms in 2023 (MAF, 2024).

Regarding the current version and the whole interface of the VetIS as a national database on animal identification and registration, more than half of the farmers, 57.97% (both categories "Strongly agree" and "Agree"), declared they were familiar with the system (Figure 1).

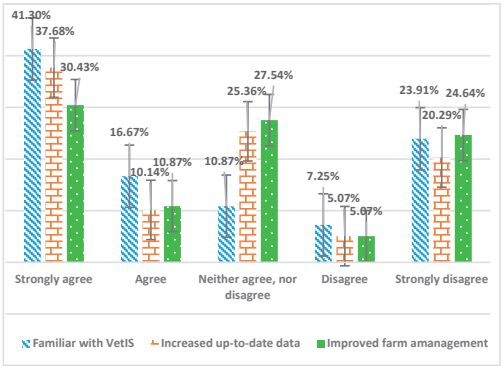


Figure 1. Respondents` distribution regarding their familiarity with the information system and the potential improvement of farm management and up-to-date data

The familiarity with the modern computerized technologies in agriculture was perceived in fact as a factor in favor of adopting the digitalization process (Blasch et al., 2020). Confronted with the new developing functionalities, 47.82% of the respondents (both "Strongly agree" and "Agree") believed that with their personal access to the system the volume of up-to-date data would increase. Similar share of the animal owners, 41.30% (both "Strongly agree" and "Agree"), were sure that these changes would lead to improved farm management for their holdings, especially with regard to traceability (Vinholis et al., 2017).

There was a positive correlation between the age of the farmers and their position on increasing the relevance of data ($R = 0.19$; $p = 0.025$) and reducing the errors in data entries in the system ($R = 0.25$; $p = 0.004$). It could be assumed that older farmers have more experience and knowledge of the importance of accurate data entry and maintaining up-to-date records which corresponded with the findings of Aubert et al. (2012) on the influence of the owners` expertise. They might also be more aware of the consequences of errors in the system and the impact they can have on their farm and business.

When analysing the livestock owners` opinion on the potential effects of the new functionalities, more than 40% (both categories "Strongly agree" and "Agree") believed that given personal access to the system would result in accurately observing the deadlines for animal identification; increased percentage of submitted applications; facilitated documentary work and reducing discrepancies in the actual number of animals, respectively (Figure 2).

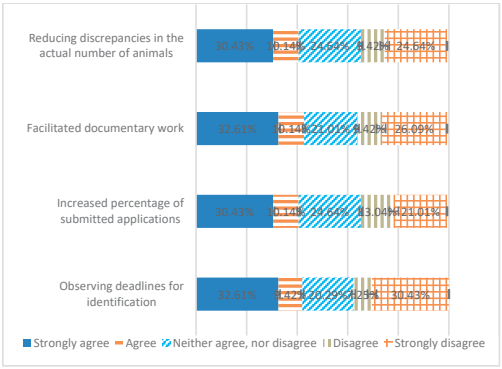


Figure 2. Farmers` opinion of the positive effects of the new VetIS functionalities

The farmers would be able to track the data entered and to correct inconsistencies in the system regarding events with their animals (birth, movement, death, slaughter). These findings come in compliance with the terms of the Commission Implementing Regulation (EU) 2021/520 of 24 March 2021 (EU, 2021) which stated that each member state should provide the opportunity to livestock owners of large and

small ruminants and swine free access to a minimum of information in the computerized database concerning their animal holdings.

There was a correlation between the number of kept animals and the expectations for compliance with the deadlines for animal identification (R = 0.20; p = 0.022) - farmers who owned more animals showed a positive attitude towards keeping the deadlines by entering data into the system by themselves.

With the modern tendencies in the field of agriculture and animal husbandry, especially the precise livestock breeding, huge volumes of data are being generated through different sources. In order to be efficiently used by different stakeholders, including regulatory bodies, business operators like industry and farmers, data should be standardized and integrated into a completed computerized database (Ngo & Kechadi, 2021). We argue that by implementing the new functionalities at national level including the livestock owners as active participants, the information system will improve its uniformness and effectiveness. It could be expected that through the means of data management of information on electronic animal identification, the processing time and generating queries by the farmers would improve (Anu & Canessane, 2017). Similar to our findings on the positive respondents` attitude towards the proposed changes appeared to be the conclusions of Goller et al. (2021) who found that the dairy farmers in Germany perceived as positive the shift to digitalization of their farm work.

Using the chi-square analysis for our parameters, we found a significant difference between the age of the farmers and the possibility to reduce the discrepancies in the real number of animals and the virtual ones ($\chi2$ = 23.603; df = 12; p = 0.023) (Table 2). The younger livestock owners` attitude appeared to be more favourable towards the opportunity to generate reports by themselves on the actual number of the animals on the farm. The age as a factor was also found significant in the adoption of innovative technologies by Granado-Díaz et al. (2024) and Shang et al. (2021) as older farmers appeared to be less likely to use new digital tools (Barnes et al., 2019). Regardless of that, as mentioned above, older farmers also believed that direct access to database will increase the accuracy of data.

Table 2. Respondents` position on the given possibility to generate queries from the information system and minimizing the discrepancies in animal numbers

Age	The oportunity for farmers to generate reports on the animals on the site would reduce the percentage of farms with discrepancies between the actual number of animals and the recorded ones in VetIS								
	Strongly Agree	Agree	Neither Agree nor Disagree	Disagree	Strongly Disagree	Total	Chi square	Df	Sig.
20-29	**50.0%**	50.0%	0.0%	0.0%	0.0%	100.0%			
30-60	**35.5%**	8.6%	26.9%	5.4%	23.7%	100.0%			
61-64	9.1%	18.2%	36.4%	18.2%	**18.2%**	100.0%	23.603	12	0.023
65+	21.4%	7.1%	14.3%	21.4%	**35.7%**	100.0%			
Total	30.9%	10.3%	24.3%	9.6%	25.0%	100.0%			

The level of education is another factor that could play an important part of the respondents` decision making regarding new technologies (Aubert et al., 2012; Li et al., 2023). Our survey showed that there was a significant difference between the educational background and the belief among the farmers that their personal access to the database would result in compliance with the legal deadlines for animal identification ($\chi2$ = 41.787; df = 16; p = 0.000) (Table 3). Higher degree of education indicated more positive attitude to the functionality giving the users opportunity to enter data on newborn animals at the establishment, which appeared in line with the findings of Shang et al. (2021) about more literate farmers being able to better comprehend modern digital applications. Significant difference was determined as well between the farm size and personal data entry regarding identification of the animals kept

($\chi 2$ = 33.728; df = 20; p = 0.028), recognizing the farm capacity as an important factor in respondents` attitude shaping (Blasch et al., 2020; Groher et al., 2020). However, the results showed that livestock owners with bigger holdings tended to disagree with the positive effect on keeping the identification deadlines, as the larger number of animals required more time and efforts for registration in the database. This appeared to be in contrast with the findings of Pierpaolia et al. (2013) and Tamirat et al. (2017) who argued that with the increase of the farm size the probability of welcoming advanced technologies was also increased. We attributed this result to the fact that many large farms had implemented their own farm management software.

Table 3. Respondents` position on the given possibility to enter data on new animals and compliance with animal identification deadlines

Education	**The oportunity for the farmer to enter data on newborn and newly marked animals would reduce violations in animal identification deadlines**								
	Strongly Agree	Agree	Neither Agree nor Disagree	Disagree	Strongly Disagree	Total	Chi square	df	Sig.
Primary school	0.0%	0.0%	40.0%	0.0%	**60.0%**	100.0%	41.787	16	0.000
Middle school	11.1%	11.1%	27.8%	0.0%	**50.0%**	100.0%			
High school	37.5%	6.3%	20.8%	7.3%	28.1%	100.0%			
Bachelor degree	**80.0%**	0.0%	0.0%	0.0%	20.0%	100.0%			
Master degree	25.0%	41.7%	8.3%	25.0%	0.0%	100.0%			
Total	33.1%	9.6%	20.6%	7.4%	29.4%	100.0%			
Number of animals kept									
	Strongly Agree	Agree	Neither Agree nor Disagree	Disagree	Strongly Disagree	Total	Chi square	df	Sig.
from 1 to 9	**50.0%**	25.0%	25.0%	0.0%	0.0%	100.0%	33.728	20	0.028
from 10 to 49	**47.4%**	7.9%	13.2%	13.2%	18.4%	100.0%			
from 50 to 99	34.3%	5.7%	17.1%	2.9%	**40.0%**	100.0%			
from 100 to 149	19.4%	16.1%	19.4%	3.2%	**41.9%**	100.0%			
from 150 to 499	26.1%	4.3%	30.4%	4.3%	34.8%	100.0%			
500 and more animals	0.0%	25.0%	25.0%	50.0%	0.0%	100.0%			
Total	32.6%	9.6%	19.3%	7.4%	31.1%	100.0%			

Once more, the educational degree and size of the animal holding were found significant for the process of managing the data entries and their tracking (Table 4). University graduates appeared to better comprehend the benefits for their farms if allowed to personally supervise the animal records and correct the errors in the database ($\chi 2$ = 28.510; df = 16; p = 0.027). Similarly, Fujimoto et al. (2022) stated that farmers with higher literacy and willing to use data for management of their establishments, showed greater probability of participating in new developments of electronic systems. Insufficient knowledge, at the same time, prevented the understanding on the benefits of adopting computer-based technologies (Takácsné György et al., 2018).

On the other hand, the size of the farm affected negatively our respondents` attitude towards the animal records control in VetIS ($\chi 2$ = 44.149; df = 20; p = 0.001). This finding appeared to be in contrast with other studies (Vinholis et al., 2017; Huang & Fu, 2023b) who determined that larger agricultural establishments were usually associated with more economic benefits, thus the farmers favoured adoption of digital technologies to improve the output.

Table 4. Respondents` position on the given possibility to generate queries from the information system and benefit the farm management and control on data

Education	The oportunity for farmers to generate reports on the animals on the site would be beneficial for farm management by allowing owners to exercise control over tracking data and correcting discrepancies in VetIS								
	Strongly Agree	Agree	Neither Agree nor Disagree	Disagree	Strongly Disagree	Total	Chi square	df	Sig.
Primary school	0.0%	0.0%	50.0%	0.0%	**50.0%**	100.0%	28.510	16	0.027
Middle school	11.1%	5.6%	33.3%	0.0%	**50.0%**	100.0%			
High school	32.6%	10.5%	29.5%	7.4%	20.0%	100.0%			
Bachelor degree	**80.0%**	0.0%	0.0%	0.0%	20.0%	100.0%			
Master degree	**41.7%**	33.3%	16.7%	0.0%	8.3%	100.0%			
Total	31.3%	11.2%	28.4%	5.2%	23.9%	100.0%			
Number of animals kept									
	Strongly Agree	Agree	Neither Agree nor Disagree	Disagree	Strongly Disagree	Total	Chi square	df	Sig.
from 1 to 9	**66.7%**	0.0%	0.0%	0.0%	33.3%	100.0%	44.149	20	0.001
from 10 to 49	**47.4%**	15.8%	15.8%	7.9%	13.2%	100.0%			
from 50 to 99	28.6%	5.7%	37.1%	5.7%	22.9%	100.0%			
from 100 to 149	29.0%	0.0%	25.8%	0.0%	**45.2%**	100.0%			
from 150 to 499	13.6%	18.2%	31.8%	9.1%	**27.3%**	100.0%			
500 and more animals	0.0%	7.,0%	25.0%	0.0%	0.0%	100,0%			
Total	31.6%	11.3%	26.3%	5.3%	25.6%	100.0%			

The results suggested that with the tendencies of growing digitalization of the agriculture, it would be beneficial for policy implementation to consider various factors that influence the farmers, as main stakeholders, to adopt the new technological developments (Granado-Díaz et al., 2024). As proposed by Kaler & Ruston (2019), livestock owners should be encouraged to use modern digital tools by providing them with access and control on the events on the farm and reduce the administrative burden (Reissig et al., 2022).

CONCLUSIONS

The presented results clearly showed that the future developments of agriculture and animal husbandry lay in the digitalization, in compliance with the international and European tendencies and regulations. To achieve the goals for sustainable and smart agriculture, governmental bodies should understand the driving behaviours of the stakeholders for successful implementation and use of digital applications and large databases, tracking animal events. The survey showed that age, education and number of animals on the farm appeared to be the most likely factors to influence the livestock owners` attitude towards the benefits and positive effects on their farm work from having real access to the animal identification database.

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ANATOMICAL FEATURES OF PHEASANT CARCASSES FROM DIFFERENT REARING SYSTEMS: A LITERATURE REVIEW

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Abstract

The anatomical features of the pheasant carcasses vary considerably depending on the rearing system, the major influences being attributed to the feeding system, sex and age. This review aims to synthesize recent data from the literature on the anatomical features of pheasant carcasses reared in natural and controlled rearing systems. This study draws on recent and relevant studies evaluating parameters such as proportions of anatomical regions and body indices according to rearing system, age and sex. Data were collected and synthesized from reputable online sources. The results show that pheasants raised in the wild reveal larger breast proportions in males, compared to those raised under controlled conditions, where was observed an important increase of subcutaneous fat and abdominal fat content. The study shows that the rearing system plays a determining role in the anatomical development of pheasants, and underscore the contributions of comparative studies on understanding its impact on carcass quality and usability. These insights can inform future strategies in pheasants rearing for economic and food production purposes.

Key words*: anatomical profile, carcass quality, pheasant, rearing system.*

INTRODUCTION

Meat is an important source of high-quality protein, essential vitamins, and minerals, and is widely consumed worldwide to meet the nutritional needs of a growing population. Amid growing concerns about health and balanced nutrition, consumer preferences are increasingly shifting towards animal products that offer high nutritional value while being low in fat (Ciobanu et al., 2022). In this context, meat from species with a distinct taste and favorable nutritional profile, such as pheasant, is gaining increasing interest (Franco and Lorenzo, 2013; Custură et al., 2019; Costache et al., 2019; Tudorache et al., 2023).

The demand for meat varies according to market trends and the eating habits of different populations. However, in recent decades, there has been a notable increase in global poultry meat consumption, at the expense of other types of meat (Godfray et al., 2018; Grigore et al., 2023). This shift is primarily driven by the greater accessibility, favorable nutritional profile, and the diverse ways in which poultry meat can be incorporated into modern diets. At the same time, growing concerns over food safety and the desire for more natural and healthier products have led to a diversification of consumer preferences, with increasing interest in alternative species such as game birds (Sarica et al., 2021; Ciobanu et al., 2024).

In this context, the poultry industry has increasingly incorporated wild bird species, such as pheasant and quail, into the human diet. These species are recognized for their complex and balanced nutritional profile (Brudnicki et al., 2012; Lorenzo et al., 2014; Franco et al., 2016; Pateiro et al., 2018; Moise et al., 2024). Not only do they offer a valuable alternative to conventional poultry, but they also provide a healthy option to replace red meats, which are often linked to an increased risk of cardiovascular and digestive diseases, primarily due to their higher fat content (Lopez-Pedruso et al., 2019; Neethling et al., 2016). Among game bird species, pheasant meat has the highest consumption rate (Bodnar et al., 2010), valued for both its nutritional benefits and distinct sensory characteristics. Specifically, the common pheasant (*Phasianus colchicus* L.) is the most widely used species for meat production due to its relatively small size, ease of rearing, and potential to yield high-quality meat (Quaresma et al., 2016).

Although the pheasant meat market is not fully structured and its availability in the commercial sector is relatively low compared to other types of meat (Chisholm et al., 2008), pheasant remains a preferred choice for many consumers due to its superior quality and nutritional value (Santos Schmidt et al., 2007). Numerous studies emphasize the nutritional potential of pheasant meat, partly explained by the species' ability to efficiently utilize native vegetation and adapt to anthropized habitats (Nuernberg et al., 2011). In addition to its favorable nutritional profile for human consumption (Nuno et al., 2018), pheasant meat is also valued for its sensory attributes, such as tenderness and juiciness (Bernacki, 2012). Furthermore, integrating this resource into the modern diet enhances the diversity of available meat products, a characteristic increasingly appreciated by today's consumers (Ciobanu et al., 2020; Boișteanu et al., 2024).

The consumption of pheasant meat from natural environments is considered advantageous both nutritionally and economically. However, the limited availability of this type of meat necessitates the exploration of alternative solutions, such as raising pheasants in intensive or semi-intensive systems, followed by their release into hunting areas or their valorization through slaughter (Sarica et al., 2021). While this strategy can contribute to biodiversity conservation and support the goals of sustainable agriculture, the quality of meat produced under controlled conditions remains a topic of debate. Studies indicate that the nutritional values and qualitative characteristics of pheasant meat are influenced by the farming system used, as well as by the management and feeding practices employed (Hofbauer et al., 2010; Franco and Lorenzo, 2013; Sarica et al., 2021). Numerous studies emphasize the importance of rearing systems not only for morphological development but also for ensuring the hygienic and nutritional quality of carcasses and derived products (Ciobotaru et al., 2024; Flocea et al., 2024). Technological factors such as feed management and environmental exposure may indirectly influence carcass composition (Matei et al., 2024).

Diversifying food preferences have led to the increasing popularity of pheasant meat among consumers in recent years, contributing to its greater availability in the commercial market (Quaresma et al., 2016). In many countries, pheasant meat production has grown visibly, attaining considerable economic and social importance, particularly in regions where hunting is a traditional activity or where pheasants are raised intensively (Pogurschi et al., 2018). As the consumption of pheasant meat has risen, public interest has also grown, not only in the quality of the meat but also in the rearing systems and production conditions (Adamski et al., 2017; Tudorache et al., 2022). In this context, the need to explore how technological factors and the applied rearing systems influence the quality parameters of pheasant meat has become increasingly evident (Postolache et al., 2015).

To date, the literature has primarily focused on determining the chemical composition and nutritional value of pheasant meat, particularly in relation to the impact of age at slaughter (Sarica et al., 1999), as well as changes induced by the reproductive system (Kuzniacka et al., 2007). Numerous studies have also explored the effect of age on the physicochemical characteristics of pheasant meat (Kuzniacka et al., 2007; Kotowicz et al., 2012). However, research on the quality of pheasant meat in relation to the rearing systems applied remains limited, highlighting the need for further investigation into this area.

In this context, the aim of this article is to analyze, through a review of the specialized literature, the anatomical characteristics of the pheasant carcass based on the rearing system applied (extensive or intensive). This approach seeks to expand existing knowledge on the influence of technological factors on the anatomical parameters of the carcass, while also serving as a foundation for future applied research in this field.

MATERIALS AND METHODS

This article is based on an analysis of available literature from academic and scientific sources, including studies published in journals, books, and research reports. The sources were selected based on their relevance to the research topic and adherence to quality criteria, such as the year of publication, the methodology used, and the impact of the findings in the field. The

review considers studies on the physical-anatomical characteristics of pheasants raised in both natural and controlled conditions, as well as research on carcass composition and yield. Sources that did not provide relevant information for the comparative analysis of pheasants raised in natural vs. controlled conditions, or those that lacked clear details on the methodologies used to evaluate carcass characteristics, were excluded.

The literature review was conducted using a systematic approach to identify trends and key conclusions from relevant studies. Qualitative synthesis techniques were applied to compare the findings from different studies, with the aim of highlighting consistencies and differences. Literature sources were extracted from academic and scientific databases, including Google Scholar, PubMed, Scopus, and Web of Science. Specific key words such as "captive-raised pheasant" , "wild-raised pheasant", "pheasant carcasses", "pheasant carcass dimensions", and "pheasant physico-chemical characteristics" were used to guide the search.

RESULTS AND DISCUSSIONS

This section presents the findings derived from the analysis of specialized literature regarding the anatomical and physico-chemical characteristics of pheasant carcasses, depending on the applied rearing system, as well as the differences between pheasants raised in natural conditions and those raised under controlled conditions.

According to Quaresma et al. (2016), pheasant is a species with an important potential for producing high-quality meat, which has sparked extensive research on both wild-raised and intensively raised specimens (Hofbauer et al., 2010; Kokoszynski et al., 2012). The literature has extensively examined various aspects of pheasant meat quality, including body structure and composition. Studies by Kuzniacka and Adamski (2010) emphasize the importance of genetic and environmental factors in shaping the musculature and skeletal structure, which are critical for producing a high-quality product. Carcass composition has also been a focus, with research by Adamski and Kuzniacka (2006) and Kokoszynski et al. (2018) showing that pheasants have a distinct distribution of muscle tissue and fat, which affects both carcass yield and the quality of the meat. Furthermore, the chemical composition of pheasant meat has been thoroughly studied (Kuzniacka et al., 2007; Brudnicki et al., 2010), revealing that pheasant meat is low in fat, contributing to its high nutritional value and unique taste. In terms of physicochemical and sensory properties, studies by Kuzniacka et al. (2007) and Hofbauer et al. (2010) demonstrate that the texture, juiciness, and tenderness of pheasant meat are particularly valued, and these qualities are influenced by the farming system and feeding practices.

Despite the superior quality of pheasant meat, its characteristics can be influenced by a range of factors, some of which are more controllable than others, as noted by Franco and Lorenzo (2013). According to Lopez-Pedrouso et al. (2019), several factors contribute to the variability in the quality parameters and composition of pheasant meat. The rearing system and feeding conditions are particularly necessary for the birds' optimal development and directly impact the carcass structure and nutritional profile of the meat. Research has shown that pheasants raised in natural or semi-intensive systems, with a varied diet, produce meat that is healthier and more nutrient-dense compared to those raised intensively (Hofbauer et al., 2010; Kuzniacka et al., 2007; Gheorghe et al., 2021; Custură et al., 2024).

Age and weight at slaughter are well-established factors that affect the texture and juiciness of meat. For instance, meat from younger pheasants tends to be more tender and easier to digest, while older birds produce meat that is tougher and less succulent (Kuzniacka et al., 2007). The sex of the bird also influences the muscle-to-fat ratio, thereby impacting the organoleptic characteristics of the meat. Male pheasants tend to yield fattier and more flavourful meat, while females generally produce leaner and more tender meat (Biesiada-Drzazga et al., 2011). Breeding conditions also have a decisive role in the health of the birds and, consequently, the quality of the meat. Research indicates that birds raised in controlled and appropriate conditions tend to have higher-quality meat, with a more favorable texture and nutritional profile for human consumption (Quaresma et al., 2016).

Pheasant rearing conditions, including factors such as age, sex, and diet, influence the anatomical characteristics of their carcasses, which complicates direct comparisons between them. Consequently, most studies on pheasant carcass anatomy focus on common parameters, such as live weight, carcass weight, carcass yield, and the proportions of the main anatomical regions derived from carcass cutting (Lopez-Pedrouso et al., 2019).

The breast is widely regarded as the most prized anatomical part of the pheasant carcass; however, its quality can also be influenced by the characteristics of the pulp (Lopez-Pedrouso et al., 2019). The proportions of the lower regions of the carcass are important, as they provide insights into the ratio between the edible and bony parts, thus playing a key role in estimating overall carcass quality (Franco and Lorenzo, 2013). Factors such as age, diet, and rearing conditions can impact the defining parameters of pheasant carcasses, particularly body weight (Tucak et al., 2008; Golze, 2010).

A comparative evaluation of pheasant carcasses raised in the wild versus those raised under controlled conditions may involve an analysis of their development based on sex, the development rate relative to age, and the physical appearance of the pheasant, considering the anatomical proportions (Tucak et al., 2008). The characteristics of the two types of carcasses, along with the key anatomical regions of interest, are outlined in Table 1 for extensive system and Table 2 for intensive system, categorized by relevant factors and the diversity of available specialized literature.

Table 1. Anatomical characteristics and particularities of pheasant carcasses obtained in extensive rearing systems by groups of relevant factors (age, gender)

Gender	Slaughter age (weeks)	LW[1] (g)	CW[2] (g)	Yield (%)	Anatomical regions			References
					Thigh (%)	Breast (%)	Wings (%)	
Female	-	910	-	-	21.51	31.41	8.77	Tucak et al. (2008)
	-	918.80	-	-	21.51*	31.41	8.77	
	16	949	691	72.8	23.2	32.7	10.6	Kokoszynski et al. (2012)
Male	-	1230	-	-	22.52	29.89	8.78	Tucak et al. (2008)
	-	1232.40	-	-	22.52	29.89	8.78	
	-	1330	870	65.5	30.90	30.10	-	Hofbauer et al. (2010)
	16	1230	903	73.4	23.9	29.9	10.4	Kokoszynski et al. (2012)
	40	1550	1360	90.1	30.59	28.77	8.36	Franco & Lorenzo (2013)

[1]LW = live weight; [2]CW = carcass weight

Table 2. Anatomical characteristics and particularities of pheasant carcasses obtained in intensive rearing systems by groups of relevant factors (age, gender)

Gender	Slaughter age (weeks)	LW[1] (g)	CW[2] (g)	Yield (%)	Anatomical regions			References
					Thigh (%)	Breast (%)	Wings (%)	
Female	-	960	-	-	20.30	26.88	7.83	Tucak et al. (2008)
	-	910	550	60.90	30.40	30.00	-	Hofbauer et al. (2010)
	12	790	-	-	-	-	-	Strakova et al. (2012)
	16	1120	-	-	-	-	-	Lukasiewicz et al. (2011)
		950	690	72.80	23.20	32.70	10.40	Kokoszynski et al. (2012)
		933	683	73.2	23.8	31.3	10.7	
		930	690	73.40	17.40	29.50	11.10	Kokoszynski et al. (2018)
	20	1040	-	-	-	17.70	-	Fernye et al. (2017)
Male	-	1144.20	-	-	20.08	26.74	8.15	Tucak et al. (2008)
	12	1020	-	-	-	-	-	Strakova et al. (2012)
	16	1230	900	73.40	23.90	29.90	10.70	Kokoszynski et al. (2012)
		1249	913	73.1	23.8	31.4	10.4	
		1240	900	72.60	18.80	28.90	11.10	Kokoszynski et al. (2018)
	32	1510	1310	84.00	32.85	24.11	7.23	Severin et al. (2007)
	40	1550	1360	90.10	30.59	28.77	8.36	Franco & Lorenzo (2013)

[1]LW = live weight.; [2]CW = carcass weight.

A comparative study of the development of pheasant carcasses raised in different systems (natural vs. controlled) was conducted by Bordei et al. (2020), who examined the differences between the two groups in terms of body weight and carcass yield. According to the authors' findings, male pheasants from natural rearing systems had visibly higher carcass weights than both females and males raised in controlled systems. This suggests a considerable influence of the rearing system on pheasant development. In contrast, female pheasants had body weights that were 15-40% lower compared to males of the same age and raised under the same conditions (Tucak et al., 2008; Golze, 2010). However, no additional comparative studies between male and female pheasants raised in controlled systems were identified. In a similar study conducted earlier, Kokoszynski et al. (2012) compared the development of pheasants from two different rearing systems: free-range and captive rearing. Consistent with the findings of Bordei et al. (2020), the authors observed that male pheasants consistently had higher body weights than females within the same system. The natural rearing system also positively impacted body weight, as both male and female pheasants raised in the wild exhibited higher body weights compared to their counterparts raised in captivity. However, the differences were not statistically significant, indicating a moderate influence of the rearing system on pheasant development. In the study on body conformation and dimensions, Kokoszynski et al. (2012) described captive-reared pheasants as having larger torsos, sternums, and chest circumferences than those raised in the wild. Additionally, female pheasants in the controlled rearing system were found to have noticeably smaller body sizes compared to females raised in the wild (Tables 3 and 4).

Table 3. Morphometric characteristics and body indices of pheasants from extensive rearing

Slaughter age (weeks)								
Parameters	Gender	6	(%)	10	(%)	13	(%)	16
Body Weight	Male	422	70.8	721	46	1053	17.6	1239
		193.6%						
	Female	367	64.5	604	36.5	825	14.9	948
		158.3%						
Body dimensions		Trunk (cm)		Chest circumference (cm)		Thigh (cm)		
	Male	20.6		28.4		13.1		
	Female	18.8		26.4		11.8		
Body indices		Massiveness (%)		Compactness (%)				
	Male	6		137.9				
	Female	5		140.4				

Source: Kokoszynski et al. (2012)

Table 4. Morphometric characteristics and body indices of pheasants from intensive rearing

Slaughter age (weeks)								
Parameters	Gender	6	(%)	10	(%)	13	(%)	16
Body Weight	Male	407	71.7	699	50.8	1054	17.2	1236
		203.7%						
	Female	366	61.2	590	36.9	808	16	937
		156%						
Body dimensions		Trunk (cm)		Chest circumference (cm)		Thigh (cm)		
	Male	20.8		28.7		13		
	Female	17.2		24.8		11.8		
Body indices		Massiveness (%)		Compactness (%)				
	Male	5.9		138				
	Female	5.4		144.2				

Source: Kokoszynski et al. (2012)

Studies by Hofbauer et al. (2010) compared the characteristics of pheasant carcasses from two rearing systems: natural and controlled. The average carcass weight of free-range male

pheasants was 875 g, while the average weight of controlled female pheasant carcasses was 555 g. These values are consistent with those reported by Tucak et al. (2008), who found average carcass weights of 830 g for free-range males and 625 g for controlled females. This research underscores the considerable differences in body size and weight between wild-raised and captive-reared pheasants.

In proportion to the weights obtained, the carcass yields of wild-raised male pheasants and captive-raised female pheasants reported by Hofbauer et al. (2010) ranged from 61% to 65.5% of the weight of the whole birds. These values are lower compared to those reported by Golze (2010), who obtained an average yield of approximately 65%, or those of Kokoszynski et al. (2012), who reported an average yield of approximately 73%. However, the weight of the muscle tissue, which is the defining characteristic of pheasant carcasses, namely the breast and leg muscles, was similar between wild-raised male pheasants and captive-raised female pheasants. This suggests that although carcass yields may vary depending on the rearing system, muscle tissue relevant to meat quality does not show important differences between pheasants raised in natural conditions and those raised in captivity.

The body weight of pheasant carcasses was studied by Lopez-Pedrouso et al. (2019), who observed that the average weight values are directly proportional to the birds' growth rate, confirming the expected trend. Regarding the age at slaughter, carcass yield can vary between 72% and 90%, with no influence from the bird's sex or diet, as reported by Kokoszynski et al. (2018).

Regarding the influence of sex on pheasant carcass composition, several studies (Kuzniacka et al., 2007; Tucak et al., 2008; Golze, 2010) have shown that sex does not impact the percentage of muscle tissue in the carcass, but it can affect the total carcass weight. Additionally, the origin of the pheasants, whether raised in the wild or in captivity, can influence the ratio of muscle tissue in the breast versus the legs (Golze, 2010), suggesting that the rearing system can affect the anatomical proportions of the carcass. According to Kokoszynski et al. (2012), pheasant body weight increases with age, and under similar growth conditions and age, male pheasants typically have a carcass weight 8-31% higher than females (Tucak et al., 2008; Kokoszynski et al., 2012; Strakova et al., 2012; Kokoszynski et al., 2018). This finding aligns with data for other game birds as well (Ozek et al., 2003).

In terms of anatomical proportions, the breast and legs are considered the most valuable parts of the pheasant carcass, making their study a focal point of much research. According to Lopez-Pedrouso et al. (2019), the proportion of the breast in pheasants ranged between 24-31%. Franco and Lorenzo (2013) observed higher breast proportions in wild-raised pheasants (29.9%) compared to the values reported by Kokoszynski et al. (2012) for captive-raised pheasants.

By evaluating the proportions of anatomical regions of pheasant carcasses by sex and rearing system, Bordei et al. (2020) identified important differences between the main anatomical regions, observing higher percentages of breast in male pheasants raised under natural conditions. The analysis of carcass composition revealed that pheasants raised under controlled conditions had a higher content of skin with subcutaneous fat and abdominal fat compared to those raised under natural conditions (Kokoszynski et al., 2018). Finally, the findings from the studies reviewed highlight the impact of rearing conditions, age, and sex on the anatomical and physicochemical characteristics of pheasant carcasses, offering valuable insights for future research and practical applications in the industry.

CONCLUSIONS

This study highlights the importance of rearing conditions in determining the physical-anatomical characteristics of pheasants and their meat quality, important for optimizing rearing technologies and for assessing the quality of pheasant-derived products. The aim of this study was to compare the physical-anatomical characteristics and carcass composition of pheasants from two different rearing systems: natural and controlled, with a focus on their influence on body weight, anatomical proportions and meat quality, in order to highlight the impact of the rearing

system on the development and nutritional value of the final product. The study revealed important differences in pheasant carcasses based on the rearing system, including variations in body weight and carcass yield. Pheasants raised in natural conditions exhibited higher body weights and a larger proportion of breast meat, while those raised under controlled conditions showed more controlled development and a higher distribution of subcutaneous and abdominal fat.

As with other game bird species, males exhibited larger body weights and sizes compared to females, regardless of the rearing system. These differences were also evident in the anatomical proportions, with male pheasants raised under natural conditions showing a higher proportion of chest meat.

The rearing system (natural or controlled) influences the development of pheasants, affecting both body weight and carcass composition. Free-range rearing appears to promote the development of more robust morphological traits, while controlled rearing results in greater fat accumulation, alongside a more uniform and controlled carcass development. Although body weight and carcass yield are important, a detailed analysis of carcass composition revealed essential differences between pheasants raised in the two systems, particularly in terms of fat content. These variations may influence the nutritional characteristics of pheasant meat and, consequently, the quality of the final product.

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REARING SYSTEMS AND THEIR IMPACT ON PRODUCTIVITY IN TURKEY FARMS: A REVIEW

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Abstract

Turkey farming is a significant sector of the poultry industry, greatly influenced by the rearing systems employed. This review explores the effects of different rearing systems intensive, free-range and extensive, on turkey production performance, including growth parameters, feed conversion efficiency, carcass quality, and meat characteristics. Existing global studies indicate that intensive systems provide the highest productivity but are associated with challenges related to animal welfare and sustainability. Conversely, extensive and organic systems improve animal welfare and meat quality but result in lower production levels and higher costs. The review also examines the role of turkey genotypes, the influence of diet and environmental conditions on performance, and the trends toward adopting sustainable systems, including the use of renewable energy and efficient waste management. Challenges, such as high costs and the need for strict regulations, are highlighted alongside opportunities for improvement through precision technologies and the integration of ecological practices. This review underscores the need for future research to identify best practices that balance productivity, animal welfare, and sustainability in turkey farming.

Key words*: animal welfare, production performance, rearing systems, sustainability, turkeys.*

INTRODUCTION

In recent years, numerous scientific publications have analyzed trends in various livestock sectors (pigs, eggs, dairy cattle:). These analyses are valuable for both market participants and decision-makers.

The present article aims to expand this body of literature by focusing on the turkey meat sector. Meat represents an important source of high quality dietary protein for a large proportion of the global population. Meat consumption is a highly debated topic worldwide, with concerns about its impact on human health, the environment, and animal welfare. Meat is a major source of protein and micronutrients, including iron, zinc, selenium, vitamin D and vitamin B_{12}, in many diets, but overconsumption has been linked to various health issues, including heart disease, stroke, cancer, and diabetes. (Ianitchi et al., 2024; Salter, 2018). The domestication of the turkey began around 1800 BC in southern Mexico and later, around 200 AD, in the southwestern United States (Speller et al., 2010). Since then, turkey domestication has undergone significant transformations. Starting in the 1940s, advancements in genetic selection (including artificial insemination), nutrition, and management practices led to increased productivity and reduced costs for turkey farmers, making turkey meat more accessible to consumers (Lasley et al., 1985; Brant, 1998; Mohan et al., 2018). The FAO's first global data collection in 1961 recorded turkey meat production at 898,000 tons. By 2023, the amount had increased to almost 5.3 million tons. This growth can be attributed to favorable production factors such as the development of the genetic and feed industries, a high degree of integration, and the use of closed housing systems. In addition, consumer preferences

have shifted. Turkey meat has a high protein content, is not restricted by religious customs, is part of the Thanksgiving tradition, and can be easily integrated into any diet. The market situation of the turkey meat sector is characterized by high nominal output prices, supported on the demand side by increasing incomes in developing countries, and on the supply side by high production costs, particularly for feed grains, energy, and labor. Turkey meat is versatile, making it a suitable substitute for other types of meat. Its distinctive flavor profile (Herkel et al., 2016) and high nutritional value recommend it as part of a healthy diet, being rich in micronutrients such as selenium, phosphorus, potassium, magnesium, zinc, and iron (Canadian Turkey, 2022). These nutrients have beneficial effects on the human immune and nervous systems. In developed countries, turkey meat is part of everyday gastronomy.

MATERIALS AND METHODS

This paper is a scientific synthesis based on a comparative analysis of specialized literature published over the past two decades, in international scientific data bases, focusing on the impact of turkey rearing systems on productive performance, animal welfare, flock health, environmental sustainability, and economic viability.

The aim of the study was to highlight the differences and convergences between the three main types of rearing systems - intensive, free-range, and extensive (free-range). The sources consulted include scientific articles from international databases such as ScienceDirect, Springer, Wiley Online Library, Scopus, and Google Scholar, as well as best practice guidelines provided by leading organizations in the field **(**Aviagen Turkeys). The selection criteria for the literature included thematic relevance, methodological rigor, data currency, and applicability to the contemporary livestock context. The extracted data were critically compared and interpreted, with an emphasis on the advantages and limitations of each rearing system, in order to provide integrated and relevant conclusions for farmers, policymakers, and researchers in the poultry sector. This methodological approach enables the formulation of scientifically grounded recommendations regarding turkey farming practices in a sustainable agricultural framework.

This review provides an integrated comparison of rearing systems, focusing not only on productivity but also animal welfare and long-term sustainability a gap not fully explored in previous reviews.

RESULTS AND DISCUSSIONS

Types of rearing systems in turkey production

1. Intensive rearing systems

The intensive rearing system for turkeys represents an industrialized method of raising these birds, characterized by maintaining large flocks in confined spaces under complete technological control of environmental conditions and feeding processes (Marchewka et al., 2013). In practice, most turkeys raised for meat are housed in enclosed buildings, typically without windows, equipped with artificial lighting systems, mechanical ventilation, permanent litter flooring (such as straw or sawdust), and automated feeding and drinking lines (Butterworth, 2019). Compared to the turkey's natural environment, these industrial shelters are poor in stimuli lacking access to the outdoors, with no opportunity for flight or pecking at vegetation and are characterized by high stocking densities (Butterworth, 2019). For example, in an intensive farm in the United States, up to 10,000 birds may be raised in a single building, with a typical density of approximately four birds per square meter (equivalent to about 0.25 m² per turkey) (Beaulac, 2018). The facilities used in these systems allow for floor rearing on permanent bedding; in some cases - especially in breeding units - special cages (batteries) adapted for artificial insemination may be used, although floor rearing remains the dominant method in meat production (Chuppava et al., 2018; Grigore et al., 2022). The foundation of the intensive system lies in maximizing production through the use of hybrid birds selected for rapid growth, high stocking densities, complete feeding programs, and strict (nutritional and veterinary) management, which

leads to an increased meat yield per unit of area (Erasmus, 2017).

1.1. *Animal welfare*

Intensive systems raise significant ethical and welfare concerns, as they constrain the natural behavior of turkeys and may cause physical or psychological suffering (Marchewka et al., 2013). According to one study, in intensive turkey farms, all three components of welfare physical condition, mental state, and the ability to express natural behavior can be negatively affected by overcrowding, unhygienic conditions, and the absence of natural environmental factors, which can even lead to the development of aggressive behaviors such as cannibalism (Beaulac et al., 2018). In industrial housing, the limited space and lack of stimulation severely restrict natural behaviors birds cannot fly or run, forage for food, or form stable groups over wide areas (Jhetam et al., 2022). High stocking density makes it difficult to avoid aggressive encounters. Similar observations were made in broiler chickens, where reduced stocking densities, compliant with EU animal welfare rules, led to improved production efficiency indices such as the European Production Efficiency Factor and the European Broiler Index (Curea et al., 2023), suggesting a trade-off between welfare-driven stocking practices and productive outcomes across poultry species. Birds may suffer injuries from pecking or collisions, especially if hierarchical or aggressive behaviors develop within large groups (Jhetam et al., 2022). To prevent cannibalism and feather pecking under such conditions, partial beak trimming (debeaking) is commonly practiced in young turkeys a painful procedure banned in many European countries (Roy, 2018). Even so, the lack of space may lead to stereotypic behaviors or chronic stress. Genetic selection for rapid growth has exacerbated welfare issues: modern turkeys reach such high body weights in a short period that they often cannot support their own weight resulting in skeletal deformities, arthritis, and leg pain. Males of heavy breeds can no longer perform natural mating (Lindenwald et al., 2021). Additionally, turkeys raised in intensive systems frequently develop skin and foot lesions due to wet litter caused by accumulated feces and condensation and airborne ammonia. These lesions (footpad dermatitis, breast burns) cause discomfort and pain (Youssef et al., 2011). Moreover, negative behaviors acquired from the inability to move freely or forage for diverse food sources impact the birds' mental state, which can be regarded as a less quantifiable but significant form of welfare impairment (Orihuela et al., 2018). In contrast, in extensive systems (such as free-range or organic systems), turkeys have access to open air and generous space, which improves certain welfare aspects. These birds can walk, peck at grass, and display natural behaviors such as dust bathing. However, there are also risks in such systems (predators, weather extremes, parasites) that can affect welfare if not properly managed.

1.2. *Genetic selection*

Genetic selection forms the foundation of performance in intensive systems. The turkeys used are meat hybrids characterized by extremely rapid growth and high yield in breast muscle. Companies such as Aviagen Turkeys and Hybrid Turkeys supply both parent and commercial lines to farms worldwide (Zampiga et al., 2020). These hybrids are unable to fly and can no longer reproduce naturally and efficiently, which makes artificial insemination necessary in breeder farms. Modern selection has led to accelerated growth (twice as fast as 50 years ago), but also to side effects: increased susceptibility to locomotor and cardiac disorders, as well as infertility in overweight males. For this reason, some recent research is testing slower-growing lines for use in alternative systems, which could offer a compromise between performance and animal welfare (Olschewsky et al., 2021).

1.3. *Productive performance*

The main goal of intensive systems is to maximize productive performance - rapid weight gain, efficient feed conversion, and high meat yield (Shehata & Hafez, 2024). In this regard, intensive systems are extremely efficient: commercial hybrid turkeys (such as the Broad Breasted White line and its derivatives) can exceed 20 kg in approximately 20 weeks under intensive conditions, fully utilizing their genetic potential through optimized nutrition and a constant microclimate

favorable to development. The feed conversion ratio (FCR) in modern turkeys is significantly improved compared to traditional populations - approximately 2.5-2.7 kg of feed are required to obtain 1 kg of body weight gain during the first months of life (Wilkanowska, 2017). These performances are difficult to achieve in free-range or extensive systems, where birds expend more energy for movement, thermoregulation, and adaptation to the environment, resulting in slower growth. A recent study showed that by 24 weeks, birds raised in semi-freedom required slightly more feed but achieved similar weights to those raised exclusively indoors; however, after this age, costs increase, and profitability drops if slaughter is delayed (Hassan et al., 2023).

In the intensive system, meat yield is maximized: muscle mass especially the breast is disproportionately developed through genetic selection, resulting in a high proportion of marketable meat per individual. However, product quality is perceived differently: some consumers prefer meat from turkeys raised slowly in free-range systems considered tastier, more textured, and lower in fat while meat from industrial systems is more uniform, higher in calories, and produced at a lower cost (Solaesa et al., 2024a). Thus, the intensive system optimizes quantity but must be carefully managed to avoid compromising quality or bird health, as stress and disease may offset productivity gains.

1.4. *Microclimate control*

Microclimate control is essential in closed housing systems, where temperature, humidity, ventilation, and lighting are fully automated. Turkey poults require an initial temperature of approximately 35-36°C, which is gradually reduced as they grow (Uemura, 2022). Ventilation plays an important role in removing excess moisture, heat, and harmful gases such as ammonia or CO_2, with systems adjusted accordingly based on the season. Optimal humidity levels range between 60-70%; deviations from this range can promote the development of pathogens or negatively affect bird comfort (Panel et al., 2023).

Modern systems include temperature, gas, dust, and humidity sensors connected to digital control panels that automatically regulate internal parameters. Lighting also influences behavior and growth: controlled photoperiods (e.g., 16 hours of light/8 hours of darkness) are used to balance development with rest (Ashabranner, 2023).

1.5. *Nutrition and feeding*

Nutrition and feeding are fully industrialized and based on compound feeds tailored to each physiological stage (starter, grower, finisher). In the first weeks, turkeys require feeds with a high crude protein content (up to 28%), which is gradually reduced to 20-24% later on, while increasing the energy supply. Rations typically include cereals (corn, wheat), soybean meal, synthetic essential amino acids (lysine, methionine), vitamins, minerals, and functional additives (coccidiostats, enzymes, probiotics), (Thesing et al., 2023). Feeding is carried out automatically using spiral or chain systems, and watering is done through automated systems (nipples, cups, adjustable bell drinkers). Monitoring feed intake is essential - any decrease may indicate a health issue. Fresh water should be available at all times, especially since an adult turkey can consume up to 4 liters per day in the finishing phase. Feed quality testing (for mycotoxins, bacteriological hygiene) is commonly used to prevent contamination and nutritional deficiencies (Valenzuela-Lino et al., 2022).

1.6. *Health risks*

Intensive turkey farming systems, due to high density and genetic homogeneity, favor the emergence and rapid spread of infectious diseases, especially respiratory ones (such as those caused by *Mycoplasma gallisepticum* or *E. coli*), particularly under conditions of inadequate ventilation and stress (Irwin et al., 2025). Stressed birds have weakened immunity, requiring frequent prophylactic treatments, including vaccines and coccidiostats. Coccidiosis is a common issue in floor-raised systems, and foot diseases impact not only welfare but also food safety (Self, 2023).

To limit these risks, strict biosecurity is required, including controlled access, thorough disinfection between flocks, and compliance with hygiene protocols. Nevertheless, the risk of outbreaks persists - as demonstrated by the H5N1 avian flu outbreak, which resulted in the

culling of over 160,000 turkeys. In extensive systems, disease spread may be slower, but contact with wild birds increases the risk of contamination (Machovcova et al., 2017).
Reducing antibiotic use, in the context of antimicrobial resistance, makes prevention through biosecurity even more critical. In conclusion, the health of flocks in intensive farms depends on continuous monitoring, strict hygiene, and rapid intervention - all of which are essential to prevent major economic losses.

1.7. *Key elements of intensive turkey rearing systems*

Stocking density is one of the most important parameters in intensive livestock management (Erasmus, 2017). Generally, in fattening houses, density varies between 3 and 4 turkeys per square meter, depending on sex and age. According to U.S. standards, it is recommended to allocate 0.23 m² per hen and 0.33 m² per tones. Excessively high stocking density can compromise ventilation, litter quality, and consequently, the health of the animals. In the European Union, there is no unified legislation, but national guidelines (such as those in Germany) recommend a maximum of approximately 40 kg/m² towards the end of the rearing period. In comparison, in extensive systems, birds have access to several square meters each, and the density is significantly lower (<1 bird/m²), while in free-range systems, an intermediate density is maintained (~2 birds/m² with controlled outdoor access) (Campbell et al., 2017).

1.8. *Biosecurity*

Biosecurity is the backbone of sanitary sustainability in industrial farms. It includes strict measures such as: controlled access, sanitary filters (showers, clean clothing), disinfection of vehicles and equipment, adherence to the “all-in/all-out” principle, controlled disposal of litter and carcasses, as well as sanitary breaks between production cycles. Daily monitoring of flock health and mortality is essential; any irregularity triggers immediate investigation. Additionally, pest control (rodents, wild birds) helps prevent the transmission of major pathogens (e.g., Salmonella, viruses). Personnel education is another key component - employees are trained not to keep birds at home and to follow strict hygiene protocols. Some farms also administer vaccines against Salmonella or use probiotics to reduce food safety risks. Well-implemented biosecurity enables the long-term health of the flock and prevents catastrophic losses (Kovács et al., 2025; Tilli et al., 2022).

2. Free-range rearing systems

Free-range turkey rearing systems represent an intermediate form of poultry farming that combines the advantages of traditional free-range methods with certain modern elements of technology and management. These systems are conceptually and practically situated between the extensive model - characterized by complete freedom of movement and use of natural resources - and the intensive system, which focuses on strict environmental control and productivity maximization (Ali et al., 2019).
According to the Food and Agriculture Organization (FAO), a free-range system involves keeping turkeys in a protected space - typically a shelter covered with a wire mesh aviary - that offers safety and comfort, while also allowing controlled access to the outdoors. At the same time, birds are provided with supplemental feed by the farmer, which distinguishes this system from fully extensive systems, where feeding relies primarily on the natural resources available in the free-range habitat.

2.1. *Animal welfare*

Free-range systems are superior to intensive ones in terms of animal welfare due to the turkeys' access to outdoor areas, natural light, and behavioral stimulation (Madzingira, 2018). Turkeys are able to express natural behaviors such as foraging, exploring the ground, and socializing in a more spacious environment, which reduces stress and harmful behaviors (Aygun et al., 2024). Additionally, footpad health is improved due to exposure to dry, natural soil, and the incidence of lesions is significantly lower (Olschewsky et al., 2021).
However, this system also comes with welfare-related risks: exposure to adverse weather conditions, predators, or overcrowding in poorly managed outdoor runs can negatively impact the birds’ comfort. Furthermore,

restrictions imposed during disease outbreaks (such as avian influenza) may temporarily cancel out the benefits of free-range systems, especially for birds accustomed to free movement. Compared to household-level systems, free-range rearing offers better supervision and veterinary care, contributing to overall improved animal welfare (Bashir et al., 2012).

2.2 *Zootechnical productivity*

Free-range systems deliver intermediate performance levels between extensive household-based systems and fully industrial intensive models. Turkeys reared under free-range conditions reach higher weights and demonstrate a better average daily gain compared to those raised in extensive systems, particularly during the first months of life, when the benefits of supplemental feeding and shelter protection are most evident. However, after 25-28 weeks of age, extensively reared turkeys may partially catch up in weight, and the economic efficiency of the free-range system begins to decline progressively (Roy, 2018).

As a result, free-range raised hens can achieve productivity close to that of those in controlled systems, albeit with a more natural feeding regime (Abba et al., 2021). The feed conversion ratio (FCR) is less favorable in free-range systems compared to industrial setups, as turkeys expend more energy for movement and thermoregulation. Consequently, feed consumption per kilogram of meat produced is slightly higher (Çürek et al., 2022). Nevertheless, for farms targeting premium product markets, this disadvantage may be offset by higher selling prices.

2.3. *Space and density*

Flock density in free-range systems is positioned between the values typical of industrial farms and those of traditional household systems. In free-range setups, European standards set a maximum of approximately 25 kg of live weight per square meter inside shelters equivalent to 2-3 turkeys/m², depending on sex and age (Aumaitre & Rosati, 2004). Each adult bird must have access to at least 4 m² of outdoor space. In practice, this translates to flock sizes of several hundred to a few thousand birds per hectare. For instance, densities of up to 4000 females/ha have been reported in free-range regimes approximately 2.5 m² per bird compared to only 1000 females/ha (10 m² per bird) in fully free-range systems (Kaya & Yildirim, 2018). Thus, birds in free-rage systems have significantly more space than those in industrial barns, though less than in household systems, where densities are negligible due to small flock sizes.

2.4. *Shelter and infrastructure*

In terms of infrastructure, free-range systems use simple shelters, often open-fronted, well-ventilated, and equipped with permanent bedding made of straw or wood shavings. These shelters may include perches where turkeys can rest at night. The shelters are directly connected to an enclosed outdoor run, where birds have free access during the day (Fa González et al., 2022). The investment required for such constructions is relatively low less than that needed for fully climate-controlled industrial barns which gives the free-range system the advantage of lower startup capital and reduced labor costs. The shelter's microclimate can be controlled to some extent through natural ventilation, protection from rain, shade in summer, or minimal heating in winter but it does not compare with the precision of the fully automated systems used in intensive farming (Mench, 2017).

2.5. *Feeding*

Feeding turkeys in free-range systems is done mostly with concentrated feed, similar in formulation to that used in intensive systems. These feeds meet the high nutritional demands of turkeys, especially in their early months (Al-Neelain, 2014). However, turkeys can supplement their diet by foraging in the outdoor paddocks, consuming grass, seeds, and insects. In the absence of feed formulated specifically for turkeys, farmers may adapt broiler feed by adding protein. By the age of 5-6 months, a turkey may consume between 20-25 kg of combined feed. When birds have access to abundant vegetation, the consumption of industrial feed can be significantly reduced, offering both economic and ecological advantages.

2.6. *Biosecurity*

Managing biosecurity in free-range turkey farming presents distinct challenges, largely due to the birds' exposure to outdoor conditions. In contrast to intensive production systems, free-range operations allow turkeys to roam in open-air spaces, heightening the risk of interaction with wild birds, rodents, and other potential pathogen carriers (Bergmann et al., 2021). To address these vulnerabilities, farms often implement secure perimeter fencing and protective netting to deter wildlife, along with pasture rotation practices aimed at minimizing the buildup of harmful organisms in the soil (Rufener et al., 2020).

Hygiene protocols in these systems commonly include footbaths at access points to pastures, routine disinfection of mobile units and shelters, and rigorous cleaning procedures for vehicles and equipment (Mulder et al., 2022). Farm personnel are instructed to change clothing and footwear before entering animal areas, and biosecurity training emphasizes the prevention of contamination between indoor and outdoor zones (Van Limbergen et al., 2018). Access to the facilities is strictly controlled, with visitors either prohibited or required to follow stringent decontamination steps prior to entry (Hartcher & Lum, 2020).

Ongoing health surveillance of the flock is vital. The active lifestyle and natural foraging habits of free-range turkeys increase the likelihood of injuries and parasitic infections, making close monitoring crucial. Any unusual mortality or clinical symptoms must trigger immediate diagnostic actions (Kittelsen et al., 2021a). Additionally, due to a higher risk of parasite exposure in outdoor settings, many farms incorporate natural anthelmintics and probiotics into their management programs to support gut health and reduce antibiotic reliance (Kralik et al., 2023).

Vaccination strategies, particularly against *Histomonas meleagridis* the causative agent of blackhead disease are essential in free-range systems, as the organism can persist in natural environments. Proper disposal of litter and carcasses is conducted away from grazing areas to avoid contamination, and downtime between production cycles is maintained to enable environmental sanitation (Van Loo et al., 2022).

Despite the inherent risks, effective biosecurity protocols tailored to the characteristics of free-range production can significantly mitigate disease threats while preserving animal welfare benefits. Ongoing staff education and the application of context-specific strategies are key to safeguarding flock health and ensuring food safety (Manning et al., 2023).

3. Extensive rearing systems

Extensive systems, also known as free-range or backyard farming systems, involve providing turkeys with generous space and constant access to the outdoors, particularly to pastures or paddocks. In Romanian tradition, this type of rearing was commonly practiced in rural households, mainly as a seasonal activity intended for self-consumption (Anna Anandh et al., 2012). From a regulatory perspective, the free-range system is defined by certain minimum criteria, particularly within the European Union. To qualify for the "free-range" label, birds must have continuous access to the outdoors for at least half of their lifespan, on vegetation-covered land, with a controlled density similar to that of the "barn" system (Jones et al., 2007). The minimum required outdoor space is at least 4 m² per bird, and exits from the shelter must total a minimum of 4 meters of opening per 100 m² of building. If the pasture extends more than 150 meters from the shelter, the presence of outdoor shelters and additional water sources is mandatory (at least 4 shelters per hectare), in order to encourage the full use of the available area (Anna Anandh et al., 2012).

3.1. *Animal welfare*

Extensive rearing systems, such as free-range or organic farming, are generally perceived as more welfare-friendly due to their allowance for natural behaviors and increased environmental complexity. Turkeys in these systems benefit from outdoor access, more space, and environmental enrichment, which improve both physical and psychological welfare indicators (Fanatico et al., 2008). Birds are able to engage in instinctive behaviors such as foraging, dust bathing, perching, and exploring, which are critical to their mental health and stress reduction (Blatchford et al., 2013).

Space availability in extensive systems allows turkeys to establish social hierarchies more naturally, reducing aggressive encounters and stress caused by overcrowding. This setup also enables more physical activity, which can prevent health issues commonly seen in intensive systems, such as skeletal deformities, obesity, and joint problems (Sossidou et al., 2011). Nevertheless, physical welfare in extensive systems depends greatly on pasture quality and environmental management - uneven terrain or poorly maintained vegetation can lead to foot injuries and stress from thermal discomfort (Petracci et al., 2010).

Outdoor access also exposes birds to climatic variations and potential predators, which can become significant stressors if not adequately controlled. Shade, shelter, and protective fencing are essential in mitigating these risks and ensuring thermal comfort and safety, particularly during extreme weather or predator activity (Hazel et al., 2020). Moreover, parasite burdens are typically higher in outdoor environments. Without effective parasite monitoring and control strategies (e.g., rotational grazing, natural anthelmintics), turkeys can suffer from infestations that impair both productivity and welfare (Singh et al., 2019).

Behaviorally, birds raised in extensive systems exhibit more species-specific actions, which are widely interpreted as signs of positive welfare. However, variations in environmental complexity, space allocation, and management quality across farms can lead to inconsistencies in welfare outcomes (De Jong et al., 2015). For example, suboptimal pasture design or lack of shelter may cause birds to cluster indoors, thus negating the potential welfare benefits of outdoor systems (Dal Bosco et al., 2016). Additionally, weather extremes and environmental stressors can exacerbate mortality and reduce comfort levels if farms are not properly equipped to offer microclimatic regulation (Sirri et al., 2010).

Feeding strategies in extensive systems also impact welfare. Access to diverse natural foraging material can enhance mental stimulation and gut health, but inconsistent nutrition due to seasonal variability may affect growth and immune response (Kittelsen et al., 2021b). Therefore, a balance between free-access foraging and supplemented, nutritionally balanced feed is necessary for maintaining optimal welfare (Matthews & Hemsworth, 2012). Despite these challenges, many welfare studies conclude that extensive rearing systems, when properly managed, result in better welfare scores across multiple indicators compared to intensive production (Louton et al., 2021).

3.2. *Breed/Genetic Line Selection*

Breeds used in extensive systems should be well-adapted to the environment and moderate growth rates. Traditional (heritage) breeds such as Standard Bronze, Narragansett, or Bourbon Red are valued for their hardiness, although they grow slower and reach lower final weights (Tutkun et al., 2018).

Many farmers use medium-growth hybrids specifically developed for free-range systems. It is recommended to source poults from certified suppliers, vaccinate them in the first days, and possibly engage in natural reproduction when working with traditional breeds (Fiorilla et al., 2023).

3.3. *Productive performance*

In extensive rearing systems - such as free-range and organic turkey production - productive performance tends to be lower compared to intensive farming. This is largely due to the increased energy demands associated with outdoor access, including greater physical activity, thermoregulation, and expression of natural behaviors (Fanatico et al., 2005). Turkeys in these environments must adapt to fluctuating weather conditions and spend more energy on movement, leading to slower growth and higher maintenance requirements than their intensively housed counterparts (Ponte et al., 2008).

Feed conversion ratios (FCR) are generally less efficient in extensive systems. While intensive operations can achieve FCRs as low as 2.5-2.7 kg of feed per 1 kg of weight gain in early growth phases, values above 3.0 are common in extensive settings, depending on factors such as breed and environmental conditions (Castellini et al., 2002). Turkeys raised in these systems are often slower-growing or traditional breeds that are selected for adaptability and hardiness rather than rapid muscle growth, which further impacts performance outcomes (Sirri et al.,

2010). For example, free-range Bronze turkeys typically reach market weights of 10-12 kg over 26-28 weeks, compared to 20 kg in just 20 weeks for intensively raised commercial hybrids (Ponte et al., 2008).

Although growth efficiency is reduced, the meat from extensively reared turkeys often possesses qualities valued by certain consumers. Increased physical activity leads to leaner muscle development, resulting in a firmer texture and more pronounced flavor - traits commonly associated with traditional or premium meat products (Sossidou et al., 2011). However, due to the absence of strong selection for breast muscle hypertrophy, as seen in industrial hybrids, breast yield is typically lower in these birds (Fanatico et al., 2007). This can reduce overall economic return unless offset by access to higher-priced specialty markets.

Seasonality and extended rearing periods also influence the cost-effectiveness of extensive systems. Turkeys are typically slaughtered later, at around 24-28 weeks of age, which increases feed, housing, and labor costs while also exposing birds to a longer period of potential environmental or health challenges (Hassan et al., 2023). Nevertheless, when well-managed and directed toward niche consumer markets, extensive systems can be financially viable. They benefit from consumer interest in welfare, environmental sustainability, and high-quality, naturally produced meat (Solaesa et al., 2024b). In many cases, consumers are willing to pay a premium for turkeys raised in systems perceived as more ethical and traditional.

In summary, while extensive systems may fall short of intensive ones in terms of growth rate and feed efficiency, they provide distinct advantages in terms of perceived meat quality, ethical farming practices, and market differentiation. These attributes make them a valuable alternative for producers targeting specialized or value-added markets (Castellini et al., 2002; Sossidou et al., 2011).

3.4. *Feeding and watering*

In extensive systems, turkey diets come from both foraging and supplemental feed. Turkeys consume grass, insects, and seeds but still require additional protein for proper development. Typically, they are fed twice a day in the morning and evening with cereals mixed with oilseed meals and mineral premixes (Spencer, 2013).

Feed can be offered using conventional feeders or traditional methods (grains scattered on the ground to stimulate natural foraging behavior). It's essential to tailor nutrition to their age: 22-28% protein during the starter phase, 20-22% during the growing phase, and 16-18% during finishing. Fresh water must be continuously available, through portable or automatic drinkers placed both indoors and outdoors, in shaded areas (Salmon, 1984). In hot weather, water temperature should be monitored; in winter, measures must be taken to prevent freezing. Supplements like probiotics or apple cider vinegar can be added to water for prophylactic purposes (Farghly et al., 2018).

3.5. *Housing and infrastructure*

Even though turkeys spend a significant amount of time outdoors, they require safe shelters for nighttime and during adverse weather conditions. It is essential to provide them with well-ventilated, dry, and sufficiently spacious housing, where they can retreat at nightfall. Ideally, perches should be installed inside, as turkeys naturally prefer to roost elevated, a behavior that gives them a sense of security against predators (Stratmann & Ringgenberg, 2023).

The shelter should be equipped with thick bedding (such as straw or wood shavings) to absorb moisture and provide comfort, which must be replaced regularly to maintain hygiene. Pop-holes (access doors) should be generously sized and installed at ground level to encourage the birds' free movement; according to standards, 4 meters of opening are recommended for every 100 m² of shelter (Glatz & Rodda, 2013). Shelters can be fixed or mobile, with a modern trend being the use of "turkey tractors" - lightweight, mobile structures moved daily across pasture, providing constant access to fresh grass while reducing soil contamination. Good practices include positioning shelters on high ground, orienting doors to the south, installing ramps or low thresholds, and equipping shelters with heat lamps for young poults during the critical starting period (Plamondon, 2003).

3.6. *Fencing and pasture management*

An important component of the extensive system is fencing, which prevents turkey escapes and protects them from predators. Metal or electric mesh fences 1.5-2 meters high are typically used, sometimes with overhead netting to prevent aerial attacks (Glatz & Rodda, 2013). In small farms, partially roofed aviaries can be arranged, while in large pastures, functional landscaping with trees, shrubs, or shade structures provides natural refuges. Pasture rotation is recommended: alternating plots prevents vegetation degradation, reduces parasite pressure, and allows soil regeneration (Glatz & Rodda, 2013).

Another best practice is to avoid raising turkeys on land recently occupied by chickens to prevent Histomonas (blackhead) infestation. Ecological soil treatments, such as lime or tannin-rich plants, can help control parasites (Callait-Cardinal et al., 2010).

3.7. *Health and biosecurity*

In free-range systems, birds are more exposed to pathogens, making prevention indispensable. Periodic deworming programs (for internal and external parasites), pasture rotation, and avoiding contact with other species (especially chickens) are standard practices (McMullin, 2022). Similar findings were reported in laying hens, where eggs from free-range systems showed improved nutritional quality but also higher microbial contamination compared to conventional systems (Nistor et al., 2015), highlighting the importance of hygiene and biosecurity in extensive poultry production. The impact of storage conditions on the final quality of poultry products, such as eggs, has also been demonstrated - with significantly better preservation of quality indices under low temperature and high humidity conditions (Gavril et al., 2013).

Vaccination is advisable, particularly against Newcastle disease, and biosecurity must be observed: controlled access, clean equipment, and hygienic shelters. Sanitary breaks and thorough disinfection should follow each flock cycle.

Daily monitoring of turkeys is essential: checking their behavior, feed intake, and general condition helps identify problems early. Practices like “finishing” turkeys in the final weeks by feeding grain-rich diets contribute to the quality of the end product (Barnes et al., 2013).

CONCLUSIONS

A comparative analysis of turkey rearing systems clearly reveals that the chosen system profoundly influences productive performance, animal health and welfare, as well as the economic and ecological sustainability of the farm. Intensive systems, focused on maximizing biological and economic efficiency, offer the highest levels of weight gain and carcass yield, supported by specialized breeds and concentrated feeding. However, this productivity often comes at the expense of animal welfare, limiting the expression of natural behaviours and increasing the incidence of pathologies associated with high stocking densities and restricted movement. On the other hand, free-range systems partially balance performance with the birds' ethological needs, providing moderate access to open air and pasture. These systems allow for more harmonious physical development, improving muscle tone and overall health, without significantly compromising productive efficiency. Beyond the technical aspects, free-range systems offer an advantage in terms of positive public perception and alignment with sustainable consumption trends. However, these systems carry higher risks related to predators, environmental diseases, and climatic conditions, requiring careful and seasonally adapted management. Economically, industrial systems offer competitive prices and profitability through volume, while alternative models become viable only if they can market their products at premium prices, targeting consumers willing to pay for quality, ethics, and sustainability. Recent trends show growing demand for turkey meat from alternative systems, and farmers who successfully communicate the advantages of their products be it flavor or ethical provenance can access real market opportunities. Overall, the choice of rearing system should be a carefully weighed compromise between productive efficiency, environmental impact, animal health, and consumer expectations. In the current context,

marked by increasing concern for sustainability and animal welfare, free-range and extensive systems seem to offer viable balanced solutions, especially in markets that value these dimensions beyond the final cost of the product.

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THE EFFECT OF CROSSBREEDING PROLIFIC PALAS EWES WITH ROUGE DE L`OUEST AND TEXEL RAMS ON IMPROVING THE QUANTITY AND QUALITY OF CARCASSES FOR LAMBS SUBJECTED TO FATTENING

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Abstract

This study evaluates the performance of F1 hybrids obtained by crossbreeding Rouge de L'Ouest x Prolific Palas and Texel x Prolific Palas breeds under controlled fattening conditions, compared to the purebred Prolific Palas sheep. The results demonstrate the clear superiority of the F1 hybrids in several key performance indicators: enhanced daily weight gain, improved feed conversion efficiency, higher slaughter yield, and a favorable carcass tissue composition characterized by increased meat content and reduced bone proportion. Additionally, the F1 hybrids showed a significant advantage in the thigh muscle index, recording values 14.5-17.3% higher than those of the Prolific Palas breed. The findings align with the diversity of high-performance meat breeds and hybrids present within the European Union, including 42 breeds and 7 hybrids in the United Kingdom, as well as numerous specialized breeds in countries such as Spain, France, Germany, Belgium, and the Netherlands. These results underscore the potential of hybrid breeding strategies to enhance meat production efficiency and carcass quality in sheep farming systems.

Key words*: hybrids, new breed, Prolific Palas breed.*

INTRODUCTION

In the European countries there are numerous meat breeds and hybrids. Thus in the United Kingdom there are 42 performance breeds and 7 types of hybrids specialised for high performance meat production and also in other countries like Spain, France, Germany, Belgium, Netherlands, in Romania there is only one meat breed, namely the Palas Meat Breed, which performs similarly to the best meat breeds in the world (Ile de France, Suffolk, Berichon du Cher and so on) (Vicovan et al., 2020; Padeanu, 2002).

It is known that lambs performance at slaughter weight varies with genotype, sex, age and fattening condition (Martynuk et al., 2001). By combining and simple crossing of 2 breeds, heavier lambs can be obtained at slaughter due to the manifestation of heterosis (Zupp, 2003).

The development of meat sheep farming is a significant focus of researchers at R.D.I.S.G.B. Palas Constanta, aimed in creating new breeds specialized for meat production.

MATERIALS AND METHODS

Research was conducted at R.D.I.S.G.B. Palas Constanta across three experimental groups, each consisting of 19 young male individuals, in three variants:

- F1 half breeds between ewes of the Prolific Palas breed and Rouge de L'Ouest rams;
- F1 half breeds between ewes of the Prolific Palas breed and Texel rams;
- Current year male offspring of the Prolific Palas breed (control group).

The groups were homogeneous and analogous in terms of body weight and age, with identical housing, feeding and care conditions provided. It was monitorized the growth evolution across all sheep's by weighing, fodder consumption, by weighing the ratio at administration and the unconsumed residues, health status of the animals,

selection of specimens for control slaughters at the end of the experiment and carcass analysis during control slaughters. The experiment lasted 62 days, feeding being done using the same ratio composed of combined feed with 15% digestible crude protein and alfalfa pellets.

Average daily weight gain, feed consumption and feed efficiency, slaughter yield, carcass tissue composition, carcass classification and feed efficiency were calculated, quality classes according to the EUROP grid, conformation and constitution indices on the live animal and on the carcass (Draganescu, 1979; Pascal, 2007).

The fattening of the three batches of lambs (19 heads/batch) was done with a combined feed that ensured a protein level of 15% DP and an energy level of 2570 kcal ME. The compound feed was administered at discretion together with 300 g alfalfa hay/head/day.

It was established the daily ingestion of feed and nutrients, the growth intensity during fattening and the results of experimental slaughter.

Two slaughter yields were determined (Tafta, 2008; Vicovan et al., 2014):

$$\text{Yield R1} = \frac{Cooled\ weight\ carcass\ (kg)}{Living\ weight\ (kg/head)}\ x\ 100$$

$$\text{Yield R2} = \frac{Cooled\ weight\ carcass\ (kg)}{Empty\ living\ weight\ (kg/head)*}\ x\ 100$$

*Empty live weight - the live weight from which the gastrointestinal mass was subtracted.

The thigh muscularity index was calculated according to Purchas' formula, quoted by Laville:

$$\text{T.M.I.} = \frac{\sqrt{\frac{G}{F}}}{LF}$$

where: G = weight of thigh muscle (g);
F = weight of femur (g);
LF = length of femur (cm).

To test the statistical significance of the differences between the mean values of the studied parameters, the variable analysis algorithm (ANOVA Single Factor) was used and TUKEY HSD TEST.

RESULTS AND DISCUSSIONS

The average daily gain achieved by the F1 Rouge de L'Ouest x Prolific Palas breed hybrids was 302.47 g, which represents an increase of 138.73 g compared to the control group, yet a decrease of 2.32 g when compared to the F1 Texel x Prolific Palas hybrids.

Research indicates that crossbreeding is considerably affected by biosynthetic activities, characterized by heightened mitochondrial activity, amplified metabolic processes and increased enzyme actions. For instance, experiments conducted by a Russian research team demonstrated that the aspartate-aminotransferase enzyme activity in crossbreed lambs rose by an average of 16.4%. These researchers concluded that there's a direct correlation between the activity levels of these enzymes and the average daily growth rates of lambs (Sanikov et al., 1981).

The obtained data showed that the differences between F1 half breed and Palas Prolific Breed are very significant in the sense that the hybrids outperform the Palas Prolific Breed by 84-85% in terms of average daily weight gain (Table 1).

Note: Out of 19 heads, 5 heads were culard - double-muscled sheep.

The p-value is 3.77684. This value is extremely small, much lower than the significance threshold of 0.05 or even 0.01. This indicates a very significant difference between the groups. Comparison between groups: The F-value (19874.12) is much larger than the F-crit value (3.1682), which confirms that there is a significant difference between at least two of the groups. The differences between the groups are statistically very significant ($p < 0.001$) -Tukey HSD Test Results.

Table 1. Weight gain of lambs under intensive fattening

Criterion no.	Genotype	n	Weight gain (g/head/ day fed) $x \pm S_x$	Duration of fattening (days)	Difference between hybrids and control batch		
					± g/head	± %	Signifiance
1	F1 Rouge de L'Ouest x Palas Prolific Breed *	19	302.47±8.50	62	+138.73	+84.73	$p < 0.001$ Very significantly
2	F1 Texel x Palas Prolific Breed	19	304.79±13.09	62	+141.05	+84.14	$p < 0.001$ very significantly
3	Palas Prolific Breed (control batch)	19	163.74±0.77	62	-	-	-

There is no significant difference between F1 Rouge de L'Ouest x Palas Prolific Breed and F1 Texel x Palas Prolific Breed ($p > 0.05$), suggesting that their performances are similar.
Also, there are highly significant differences ($p<0.001$) between Palas Prolific Breed and the other two groups. These results indicate that Palas Prolific Breed has significantly lower performance compared to the two F1 hybrid breeds (Table 2).
To improve performance, the use of F1 hybrids in genetic improvement programs or breeding strategies could be considered. This means that there is a real variation between the performances of the three groups analyzed, and not just a random difference.
In terms of specific energy consumption, F1 Texel × Palas Prolific lambs had the highest value, with 3899.8 kcal/head/day, followed by Palas Prolific lambs (3816.3 kcal/head/day) and F1 Rouge de L'Ouest × Palas Prolific crossbreeds, which consumed 3699 kcal/head/day. Although the differences in energy intake are not very large between groups, it is observed that the hybrids used the energy from the feed rations administered for the growth and fattening process more efficiently. In terms of digestible crude protein, daily consumption ranged between 311.29 g and 318.63 g, with the highest value recorded in F1 Rouge de L'Ouest × Palas Prolific hybrids and the lowest value in F1 Texel x Rasa Prolific hybrids, which may suggest a better use of nutrients for muscle mass development.
The average daily gain per head and day further highlights the superiority of the hybrids over the Prolifica Palas breed. Thus, F1 Rouge de L'Ouest × Palas Prolific recorded an average daily gain of 303.0 g, and F1 Texel × Palas Prolific a similar value, of 304.8 g. In contrast, lambs from the Prolifica Palas breed had a significantly lower gain, of only 164 g/day, indicating a reduced capacity to utilize feed for weight gain.
The efficiency of feed conversion administered confirms the genetic advantage of the hybrids. They had values of 237.0 g (F1 Rouge de L'Ouest × Palas Prolific) and 234.5 g (F1 Texel × Palas Prolific), which indicates a superior conversion of feed into body weight. On the other hand, lambs from the Prolifica Palas breed had a lower efficiency, with an index of 185.0 g, which confirms that this breed requires more feed to achieve a similar growth rate to that of hybrids.
These results clearly demonstrate the beneficial impact of hybridization on growth performance and the efficiency of the use of feed administered in their diet. Both F1 Rouge de L'Ouest × Palas Prolific and F1 Texel × Palas Prolific benefited from the heterosis phenomenon, recording higher weight gains and more efficient feed conversions compared to the Prolific Palas breed. In this context, the use of hybrids in intensive fattening systems represents an advantageous strategy, contributing to the optimization of meat production and maximizing economic profitability in specialized farms (Table 3).

Table 2. Tukey HSD Test Results

Group 1	Group 2	Mean Difference	p-value	Significance
F1 Rouge de L'Ouest x Palas Prolific Breed	F1 Texel x Palas Prolific Breed	Very small	> 0.05	No
F1 Rouge de L'Ouest x Palas Prolific Breed	Palas Prolific Breed	Large	< 0.001	Yes
F1 Texel x Palas Prolific Breed	Palas Prolific Breed	Large	< 0.001	Yes

Table 3. Feed conversion efficiency of F1 hybrids vs Palas Prolific Breed

Criterion no.	Genotype	Specific consumption per head/day/fed			Feed conversion efficiency* (g)
		Kcal	PBD (g)	Daily average gain (g)	
1	F1 Rouge de L'Ouest x Palas Prolific Breed	3699	318.63	303.0	237.0
2	F1 Texel x Palas Prolific Breed	3899.8	311.29	304.8	234.5
3	Palas Prolific Breed	3816.3	317.02	164	185.0

Note: *Feed conversion efficiency = growth gain achieved for consumption of 1 kg dry matter.

The slaughter yield, expressed as R1, ranged between 46.87% and 48.80% in the F1 hybrid groups, while for the Prolific Palas breed, it was recorded at 44.63%. Although the differences among the hybrid groups were statistically insignificant ($p > 0.05$), the slaughter yield R1 was higher in the hybrids by 5.01% to 9.34% compared to the purebred Prolific Palas sheep, with these differences being statistically significant ($p < 0.05$).

Similarly, the slaughter yield R2, which accounts for a more refined assessment of carcass efficiency, varied between 51.32% and 56.85%. The hybrid groups demonstrated an increased slaughter yield R2 by 6.06% to 10.8% in comparison to the Prolific Palas breed, confirming a significant advantage ($p < 0.05$). These findings highlight the positive impact of crossbreeding on meat production efficiency, as the F1 hybrids exhibited superior slaughter yields, likely due to improved carcass conformation and muscle development inherited from their sire breeds (Table 4).

The meat content in the carcass ranged between 75.29% and 75.52% in the F1 hybrid groups, whereas for the Prolific Palas breed, it was notably lower at 68.46%. Although the differences between the two hybrid groups were statistically insignificant ($p > 0.05$), both hybrids exhibited a significantly higher meat content compared to the Prolific Palas breed, with increases ranging from 9.98% to 10.31% ($p < 0.05$). In addition to the increased meat proportion, the tissue composition analysis revealed variations in bone content. The F1 hybrids displayed a bone content ranging from 22.55% to 25.19%, while the Prolific Palas breed had a higher bone proportion of 28.24%. This indicates that the hybrid groups had a reduced bone content by 7.99% to 8.92%, suggesting a more favorable meat-to-bone ratio. These findings emphasize the genetic advantage conferred by crossbreeding in enhancing carcass quality, as the hybrids not only exhibited a greater proportion of meat but also a lower skeletal proportion, which is desirable for meat production (Table 5).

Table 4. Slaughter yield

Criterion no.	Genotype	Y1%	Y2%	Difference between hybrids and control batch (±%)		Signifiance
				Y1	Y2	
1	F1 Rouge de L'Ouest x Palas Prolific Breed	46.87 ± 1.15	54.41 ± 1.82	+5.01	+6.06	$p < 0.05$ significantly
2	F1 Texel x Palas Prolific Breed	48.80 ± 1.75	56.85 ± 2.05	+9.34	+10.8	$p < 0.05$ significantly
3	Palas Prolific Breed (control batch)	44.63 ± 0.97	51.32 ± 2.15	-	-	-

Table 5. Tissue structure

Criterion no.	Genotype	Tissue structure, %			
		muscle	bone	fat	meat
1	F1 Rouge de L'Ouest x Palas Prolific Breed	61.93± 6.2790	22.55± 0.3014	13.36± 0.105	75.32
2	F1 Texel x Palas Prolific Breed	66.37± 2.0788	25.19± 0.6463	9.05± 0.3100	75.52
3	Palas Prolific Breed	53.02± 4.2500	28.24± 0.8563	15.44± 0.2414	68.46

The comparative analysis of tissue structure and carcass yield among the three genotypes highlights the superiority of F1 crossbreeds over the purebred Palas Prolific. Both F1 Rouge de L'Ouest × Palas Prolific and F1 Texel × Palas Prolific exhibited significantly higher muscle percentages (+8.91% and +13.35%, respectively) and carcass yields (+6.86% and +7.06%) compared to the control group, along with notable reductions in bone and fat content. These differences were statistically significant ($p < 0.05$), confirming the positive effect of crossbreeding on carcass quality traits. When comparing the two F1 crossbreeds, F1 Texel × Palas Prolific showed slightly better values in terms of muscle mass and fat reduction; however, the differences between the two hybrid groups were not statistically significant ($p > 0.05$). This suggests that both crossbreeding strategies are effective and yield comparable improvements in carcass composition, offering viable genetic alternatives for enhancing meat production in sheep (Table 6).

Table 6. Comparative differences between groups - tissue structure and carcass yield

Comparison between groups	Muscle (%)	Bone (%)	Fat (%)	Carcass yield (%)	Significance
F1 Rouge x Palas Prolific vs. Palas Prolific	+8.91	–5.69	–2.08	+6.86	Significant difference ($p < 0.05$)
F1 Texel x Palas Prolific vs. Palas Prolific	+13.35	–3.05	–6.39	+7.06	Significant difference ($p < 0.05$)
F1 Rouge x Palas Prolific vs. F1 Texel x Palas Prolific	–4.44	+2.64	+4.31	–0.20	Not significant ($p > 0.05$)

The carcasses of the F1 hybrid groups - Rouge de L'Ouest × Palas Prolific and Texel × Palas Prolific - were classified within the U2-3 quality class based on the EUROP grading system. This classification indicates superior conformation with well-developed musculature and an optimal level of fat cover, making them more desirable for meat production.

In contrast, the carcasses of the purebred Prolific Palas sheep were categorized in the R2-3 quality class, signifying a lower degree of muscle development and a slightly less favorable conformation compared to the hybrid groups. This difference underscores the impact of crossbreeding in enhancing carcass quality, as the F1 hybrids benefited from the superior meat-producing traits of the Texel and Rouge de L'Ouest sire breeds. These results highlight the genetic improvements achieved through crossbreeding, leading to better carcass classification and overall meat yield potential (Table 7).

Table 7. Classification of carcass quality classes

Criterion no.	Genotype	Quality classes according to EUROP grid
1	F1 Rouge de L'Ouest x Palas Prolific Breed	U2-3 (very good, low or medium fat carcass)
2	F1 Texel x Palas Prolific Breed	U2-3 (very good, low or medium fat carcass)
3	Palas Prolific Breed	R2-3 (good, low or medium fat carcass)

The thigh muscularity index recorded for the Prolific Palas breed was 0.510, where as for the F1 hybrid groups, the values ranged between 0.655 and 0.683. Specifically, the F1 Texel × Prolific Palas hybrids exhibited a thigh muscularity index of 0.655, while the F1 Rouge de L'Ouest × Prolific Palas hybrids reached a higher value of 0.683. These results indicate a significant improvement in muscularity among the hybrid groups compared to the purebred Prolific Palas sheep. More precisely, the thigh muscularity index was 28.43% higher in the F1 Texel × Prolific Palas group and 33.92% higher in the F1 Rouge de L'Ouest × Prolific Palas group than in the Prolific Palas breed. This suggests that crossbreeding with Texel and Rouge de L'Ouest rams positively influenced the development of muscle mass in the offspring, likely due to the superior genetic potential for meat production of these terminal sire breeds (Table 8).

Table 8. Thigh muscle index of F1 hybrids compared to Palas Prolific Breed

Criterion no.	Genotype	Thigh Muscularity Index (T.M.I.) after Purchas formula
1	F1 Rouge de L'Ouest x Palas Prolific Breed	0.683 ± 0.2344
2	F1 Texel x Palas Prolific Breed	0.655 ± 0.2168
3	Palas Prolific Breed	0.510 ± 0.0199

CONCLUSIONS

The study highlights the distinct advantages of F1 hybrids (Rouge de L'Ouest x Prolific Palas and Texel x Prolific Palas) over the purebred Prolific Palas breed in terms of key performance indicators in lamb fattening. Notably, the F1 hybrids demonstrated superior weight gain, feed conversion efficiency, and carcass yield, with an increased meat proportion and reduced bone content compared to the Prolific Palas breed.

It is demonstrated an improved growth and feed efficiency, the F1 hybrids achieved a significant increase in daily weight gain, surpassing the Prolific Palas lambs by up to 85%. Their feed conversion efficiency was also notably higher, confirming the efficiency of these crosses in utilizing feed for growth.

Both F1 hybrid groups exhibited higher slaughter yields (R1 and R2), with better conformation, resulting in more muscle and less

bone in the carcass. The meat content was higher by 6.83-7.06 percentage points compared to the purebred Prolific Palas lambs.

The hybrids also showed a significant improvement in the thigh muscularity index, indicating enhanced muscle development. Furthermore, the carcasses of F1 hybrids were classified in a higher EUROP quality class (U2-3), reflecting superior meat production traits, in contrast to the R2-3 classification of the Prolific Palas breed.

The results underscore the value of crossbreeding with high-performance terminal sire breeds like Rouge de L'Ouest and Texel in genetic improvement programs for sheep meat production. The genetic benefits observed from heterosis in the F1 hybrids suggest that this strategy could optimize productivity in specialized farms and improve economic profitability.

In conclusion, the hybridization of the Prolific Palas breed with Texel and Rouge de L'Ouest rams holds significant promise for improving lamb fattening performance, carcass quality, and overall meat production efficiency. These F1 hybrids are expected to play a crucial role in the development of a new meat breed that could adapt well to specific environmental conditions, particularly in arid areas.

Future breeding programs focusing on maintaining these advantageous traits through successive generations will likely lead to further enhancements in meat sheep farming.

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COMPARATIVE STUDY ON THE INTENSIVE FATTENING OF PALAS MERINO LAMBS AND PALAS MERINO × PALAS MEAT BREED HYBRIDS

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Abstract

Given the rising interest in sheep meat, this study aims to explore fattening technologies and improve meat production by crossing local breeds with specialized meat breeds. Research was conducted at R.D.I.S.G.B. Palas-Constanța to test the fattening performance of F1 hybrids (Palas Meat Breed x Palas Merino) compared to Palas Merino lambs. Two groups were intensively fattened for 100 days using granular feed with 87.9% dry matter, 2570 kcal, and 16% digestible crude protein. Initial body weights were similar (20.48-22.78 kg), with no significant differences ($p<0.05$). At the end, body weights ranged from 36.14 kg to 45.07 kg, with hybrids weighing 24.71% more ($p<0.01$). Average daily gain was 157-223 g, 42.04% higher in hybrids. Specific energy and protein consumption were 27.66% and 27.54% lower, respectively, in the F1 hybrid group. Conformation and constitution indices were superior in hybrids compared to Palas Merino lambs.

Key words*: body weight, Merino, meat, hybrids, yield.*

INTRODUCTION

Romanian research in the field of sheep breeding has long focused on improving meat production in local sheep breeds. This emphasis arises from the fact that attempts to breed and adapt specialized meat-producing sheep imported from other countries have not realized the expected results in Romania. These attempts have led to significant losses due to high mortality rates and a dramatic decline in reproductive parameters (Vicovan et al., 2009).

To enhance meat production quality in Romanian sheep breeds, the solution lies in developing F1 and R1 crossbreeds specifically for meat production. This involves using local sheep breeds crossed with genetically improved meat-producing breeds, thereby harnessing the benefits of heterosis (hybrid vigor), which results in increased vitality and performance in hybrid offspring compared to the parental lines (Dima et al., 2006; Draganescu, 2006; Pascal et al., 2009).

Given the current economic context, sheep breeding and production must be aligned with market demands while maintaining environmental sustainability. There is a growing trend toward refining rearing and fattening technologies for lambs, with a focus on developing breeds and genetic lines with superior meat production traits. Additionally, efforts are being made to produce high-quality carcasses that meet both quantitative and qualitative standards (Gutierrez et al., 2005; Kukovics et al., 2013).

Furthermore, it is necessary to reassess the profitability of sheep farming by creating highly productive crossbreeds. In developed countries, such as the United Kingdom, France, Spain, New Zealand, Australia and the United States, the preferred method involves industrial crossbreeding to obtain first-generation hybrids (F1) with superior carcass quality parameters (Pascal et al., 2009).

To support the advancement of meat-oriented sheep production, strategic emphasis must now be placed on integrating these genetic improvements into scalable and economically viable production systems. This involves not only the selection of superior genotypes but also

the adaptation of flock management practices that optimize reproductive efficiency, health status, and feed utilization across diverse production environments in Romania.

Equally important is the need to strengthen breeder networks and data collection infrastructures, which can facilitate the monitoring of performance indicators and accelerate genetic gain across herds. Partnerships between research institutions, breeding associations, and commercial farms are essential in this regard, enabling the dissemination of best practices and the alignment of genetic progress with market expectations.

Furthermore, Romania's geographic and ecological diversity offers an untapped opportunity for the regional specialization of meat sheep systems. Zones with favorable pasture availability and access to markets could be designated for intensive lamb fattening operations, while more extensive systems may be optimized for dam lines and hybrid lamb production. Developing such regional strategies would enhance both production efficiency and environmental sustainability.

Investing in farmer training, technical advisory services, and value chain development including slaughter, processing, certification, and marketing will be critical for transforming genetic potential into measurable economic outcomes. By fostering an integrated approach that combines genetics, technology, and market orientation, Romania can position itself as a competitive player in the European and international lamb meat sectors.

MATERIALS AND METHODS

The research was conducted at Research and Development Institute for Sheep and Goat Breeding Palas-Constanta on two groups, each consisting of 20 males from the current year, as follows: the first group it is consisted in Palas Merino lambs and the second group - F1 (Palas Meat Breed × Palas Merino) lambs.

The study focused on evaluating the growth performance of these groups by assessing the following aspects: weight gain, nutrient intake and the calculation of body conformation and constitution indices.

Body measurements on live animals were performed using a zoometer, caliper and measuring tape on shorn animals: the hip joint width was measured with a caliper between the coxofemural joints; the perimeter of the gigot was measured with a tape passing over the knee joint and the rump point; the length of the gigot was measured with a tape stretched along the inner side of the right hind limb, from the ischiopubic symphysis to the middle of the tibiometatarsal joint. Before taking this measurement, the animal was slightly lifted by the tail to relax the hind limbs.

Based on these body measurements, the following constitution indices were calculated:

- Compactness Index of the Gigot (according to Laville et al., 2002):

$$\text{C.I.G.} = \frac{Width\ of\ coxofemural\ joints}{Length\ of\ gigo}\ x\ 100$$

- Muscularity Index of the Gigot (Vicovan et al., 2009):

$$\text{M.I.G.} = \frac{Perimeter\ of\ the\ gigot}{Length\ of\ gigot}\ x\ 100$$

For the intensive lamb fattening phase, which lasted 100 days, a pelleted feed was used with the following nutritional content: 2,570 kcal/kg energy, 16% crude protein, 3.5% crude fat and 8.5% crude fiber, in accordance with nutritional standards. The fattening control was carried out by daily weighing of the administered feed, with leftover feed being collected every 3-4 days.

To test the statistical significance of the differences between the parameter values studied, one-way ANOVA (single-factor analysis of variance) was used.

RESULTS AND DISCUSSIONS

At the beginning of the experimental period, the body weight of the individuals ranged between 20.48 kg and 22.78 kg (Table 1).

The F1 crossbred lambs (Palas Meat Breed x Palas Merino) had an average body weight of 22.79 kg at the beginning of the fattening period, and at the end of the fattening period, their average body weight was 45.07 kg, achieving an average daily weight gain of 223 g. In the batch of lambs from the Palas Merino breed, the initial body weight was 20.48 kg and the final average body weight was 36.14 kg, resulting in an average daily weight gain of 157 g.

Table 1. The evolution of lambs body weight during the fattening period

Genotype	Body weight (kg/head)				Average daily gain (g/head)	
	At the beginning of the fattening		At the end of fattening			
	$X \pm s_{\bar{x}}$	V%	$X \pm s_{\bar{x}}$	V%	$X \pm s_{\bar{x}}$	V%
F1 crossbred lambs (Palas Meat Breed × Palas Merino	22.79± 1.24	24.60	45.07 ± 1.44	14.45	223± 5.98	11.98
Palas Merino lambs	20.48± 0.63	13.56	36.14 ± 0.83	10.22	157± 6.04	17.16
Difference between F1 crossbreed and Palas Merino average daily gain	g				+66	
	%				+42.04	
Significant difference	p < 0.001 (very significant difference)					

The observed difference in average daily gain (average daily gain) between the F1 crossbreeds and the Palas Merino breed is both substantial and statistically highly significant. Specifically, the F1 lambs recorded an increase of +66 grams/day, representing a +42.04% improvement over the performance of the purebred Palas Merino lambs. The very significant p-value ($p < 0.001$) confirms that this difference is not due to random variation but is instead attributable to the genetic advantage conferred by crossbreeding, likely through heterosis (hybrid vigor).

The ANOVA results highlight a very significant difference between the two groups analyzed: F1 crossbred (Palas Meat Breed × Palas Merino) and Palas Merino lambs. The F1 crossbred group achieved a higher average performance (223 g) compared to the Palas Merino (157 g), indicating a substantial advantage in productivity. Furthermore, the statistical test confirms that this difference is not due to random variation, as the p-value is extremely low (3.66), far below the 0.05 significance threshold. Additionally, the calculated F-value (322.67) far exceeds the critical F-value (4.098), reinforcing the robustness of the observed differences. These findings strongly suggest that F1 hybrids significantly outperform purebred Palas Merino sheep. This result underscores the potential benefits of hybrid breeding programs, which could be leveraged to enhance productivity and efficiency in sheep farming. Given these insights, incorporating F1 crossbreds into breeding strategies may lead to improved economic returns and enhanced genetic performance, making them a valuable choice for sustainable sheep farming.

This result highlights the effectiveness of using F1 crossbreeds in meat-oriented sheep production systems. The improved growth performance has direct economic implications, including shorter fattening periods, better feed efficiency, and increased carcass yield, all of which contribute to enhanced profitability for commercial producers. Such findings support the strategic use of controlled crossbreeding as a viable tool for improving meat production traits in Romanian sheep populations.

The data obtained in the present experiment are comparable to other experiments, where the effects of crossbreeding Transylvanian Merino ewes with rams from various breeds were examined, including Suffolk, Merinofleisch and Berrichon du Cher (Maier, 1975). The groups subjected to fattening showed relatively similar growth increases, which is mirrored in the present research, where the growth performance of the different experimental groups was closely comparable.

The average feed consumption per head, per day, and the specific consumption (feed intake per kg of live weight gain) are presented in Table 2, with the data expressed both per head per day and per kg of live weight gain.

F1 crossbred lambs (Palas Meat × Palas Merino) consumed, on average, 1.06 kg of feed per head per day, while Palas Merino lambs consumed 1.03 kg. This suggests that Palas Merino lambs had slightly lower feed consumption compared to the F1 crossbred lambs. Regarding feed consumption per kg of weight gain, F1 crossbreds required 4.75 kg of feed to gain 1 kg of weight, whereas Palas Merino lambs required 6.56 kg, which is 38.10% higher. This is an indicator of feed conversion efficiency, and the data suggest that F1 crossbreds have better feed conversion, needing less feed to gain the same amount of weight. F1 crossbreds consumed, on average, 2724 kcal of metabolizable energy per head per day, while Palas Merino lambs consumed 2647 kcal. The difference in energy consumption is relatively small, but F1 crossbreds appear to have slightly higher consumption, indicating a greater energy requirement for their growth. Regarding metabolizable energy per kg of live weight gain, F1 crossbreds required 12197 kcal to gain 1 kg

of weight, compared to 16860 kcal for Palas Merino lambs, with the latter having 38.23% higher specific energy consumption. This highlights a better energy utilization efficiency in F1 crossbreds, as they need less energy to achieve the same weight gain (specific consumption). F1 crossbred lambs consumed 170 g of digestible crude protein per head per day, while Palas Merino lambs consumed 165 g. The difference is minor (only 3.03%), but F1 crossbreds appear to benefit from a slightly higher protein intake, which may support faster muscle tissue development. Regarding digestible crude protein per kg of live weight gain, F1 crossbreds need 760.5 g of digestible protein to achieve 1 kg of live weight, while Palas Merino lambs need 1049.6 g, which is about 38.02% higher compared to F1 crossbreds. This suggests that F1 crossbreds have greater efficiency in utilizing protein for weight gain (Table 2).

Table 2. Feed and nutrient consumption in the lamb lots undergoing fattening

Genotype	Combined feed (kg)		Metabolizable energy (Kcal)		Digestible crude protein (g)	
	Per head and day	Per kg of weight gain	Per head and day	Per kg of weight gain	Per head and day	Per kg of weight gain
F1 crossbred lambs (Palas Meat Breed × Palas Merino)	1.06	4.75	2724	12197	170	760.50
Palas Merino lambs	1.03	6.56	2647	16860	165	1049.60

After the completion of the growth and fattening process, the main body composition and conformation indices of the fattened lambs were determined, focusing on the development of the hindquarters based on genotype.

For the width of the coxofemoral joints, F1 crossbred lambs had an average of 22.53 ± 2.16 cm, with a coefficient of variation (V%) of 16.57, while the Palas Merino lambs had slightly wider joints, measuring 23.22 ± 2.13 cm, with a lower variation of 15.90%. This suggests that Palas Merino lambs have a slightly wider hip structure.

Regarding the perimeter of the gigot, both groups had similar measurements: F1 crossbred lambs had 55.22 ± 3.88 cm, while Palas Merino lambs had 55.17 ± 3.53 cm. The variation for F1 crossbreds was 12.16%, while for Palas Merino lambs it was slightly lower at 11.07%, indicating a more consistent circumference in the Palas Merino lambs.

For the length of the gigot, F1 crossbred lambs had an average length of 25.83 ± 2.17 cm, which was 1.89% larger than the length of the Palas Merino lambs' gigot, which measured 25.35 ± 2.15 cm. The variation in length was greater for F1 crossbreds (14.52%) compared to Palas Merino lambs (4.72%), indicating that the length of the hind limbs was more variable in the F1 crossbreds.

The compactness index of the gigot (C.I.G.), which measures the proportional development of the hindquarters, was 87.22 ± 5.79 for F1 crossbreds, with a coefficient of variation of 11.49%. For Palas Merino lambs, the C.I.G. was slightly higher (by 4.37%), measuring 91.59 ± 5.25, with a lower variation of 9.93%, indicating that the hindquarters of the Palas Merino lambs were more compact.

Finally, the muscularity index of the gigot (M.I.G.), which reflects the degree of muscular development of the hindquarters, was 213.78 ± 9.98 for F1 crossbreds, with a variation of 8.08%. In comparison, Palas Merino lambs had an M.I.G. of 217.63 ± 8.96, with a slightly lower variation of 7.12%, suggesting that Palas Merino lambs had slightly greater muscular development in the hindquarters, with more compactness.

Overall, while the F1 crossbred lambs presented slightly larger and longer gigots, the Palas Merino lambs demonstrated a more compact and consistent morphology of the hindquarters, particularly in terms of muscularity and compactness indices (Tabel 3).

Table 3. Body measurements and morphological indices on live animals, with reference to hindquarter development based on genotype

Genotype / Specification	F1 crossbred lambs (Palas Meat Breed × Palas Merino)		Palas Merino lambs	
	$X \pm s_{\bar{x}}$	V%	$X \pm s_{\bar{x}}$	V%
Width of coxofemural joints (cm)	22.53±2.16	16.57	23.22±2.13	15.90
Perimeter of gigot (cm)	55.22±3.88	12.16	55.17±3.53	11.07
Length of gigot (cm)	25.83±2.17	14.52	25.35±2.15	4.72
Compactness index of the gigot (C.I.G.)	87.22±5.79	11.49	91.59±5.25	9.93
Muscularity index of the gigot (M.I.G.)	213.78±9.98	8.08	217.63±8.96	7.12

CONCLUSIONS

Crossbreeding local breeds with specialized meat breeds has proven to be an effective method for improving meat production both quantitatively and qualitatively. This demonstrates the benefits of using heterosis, which helps achieve higher yields in lamb growth and fattening.

F1 hybrid lambs (Palas Meat Breed x Palas Merino) had a final body weight 24.71% higher compared to lambs from the Palas Merino breed. During the fattening period, hybrid lambs had an average daily gain 42.04% higher than lambs from the Palas Merino breed, with very significant differences ($p<0.001$).

The lower specific consumption of energy and protein in F1 hybrid lambs resulted in lower values of metabolizable energy and digestible protein. Energy consumption was 27.66% lower, and digestible protein consumption was 27.54% lower compared to lambs from the Palas Merino breed.

The evaluation of the main body structure and conformation indices in lambs from both groups showed higher values for F1 hybrid lambs compared to lambs from the Palas Merino breed, indicating greater suitability for meat production.

These conclusions suggest that crossbreeding the Palas Merino breed with specialized meat breeds can lead to significant improvements in growth and fattening performance in lambs, with important economic benefits for farmers.

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BALANCING PRODUCTIVITY, WELFARE, AND SUSTAINABILITY IN LAYING HEN FARMING: A REVIEW OF REARING SYSTEMS

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Abstract

Rearing systems play a crucial role in shaping the productivity, welfare, and sustainability of laying hen farming. This review examines the effects of different rearing systems - cage systems (conventional and enriched), barn systems, free-range, and organic systems - on key production parameters, including egg yield, feed conversion efficiency, egg quality, and hen welfare. Studies at the global level reveal that conventional cage systems achieve the highest productivity but often raise concerns about animal welfare. Conversely, free-range and organic systems promote better welfare and consumer trust in product quality but are associated with lower productivity and higher production costs. This review also analyses the impact of environmental factors, genotype selection, and management practices on laying hen performance within each system. Additionally, it discusses emerging trends such as the adoption of enriched cages, precision farming technologies, and sustainable feed alternatives, as well as challenges like disease management and compliance with increasingly strict animal welfare regulations. By synthesizing current global research, this paper aims to highlight best practices and provide a roadmap for balancing productivity, animal welfare, and environmental sustainability in laying hen farming.

Key words*: animal welfare, egg production, laying hens, rearing systems, sustainability.*

INTRODUCTION

In modern poultry farming, achieving an optimal balance between productivity, animal welfare, and sustainability is a growing concern across the industry. As consumer awareness and legislative pressure increase, the scrutiny over how laying hens are housed has intensified (Mench et al., 2011; Harlander-Matauschek et al., 2015). Conventional battery cages, once the standard, are now increasingly challenged due to their impact on animal welfare, particularly due to the limitation they impose on natural behaviors such as perching, dustbathing, and nesting (European Commission, 1999; Lay et al., 2011).

Alternative systems - such as enriched cages, barn systems, free-range, and organic housing - have emerged as potential solutions. While these systems often result in improved behavioral outcomes and lower stress for hens, they also present significant trade-offs in terms of productivity, health risks, and environmental footprint (Rodenburg et al., 2008; Singh et al., 2017). Organic and free-range systems, for instance, align better with public values and ethical expectations but are generally associated with increased costs and lower efficiency per unit of production (Kijlstra & Eijck, 2006).

Moreover, research highlights that performance outcomes across these systems are influenced not only by housing type but also by genotype, environmental conditions, and management strategies (Tauson, 2005; Rodenburg et al., 2013). The development of precision livestock farming technologies, including sensor-based health monitoring and automated feeding, has opened new opportunities to improve both welfare and efficiency across all rearing systems (Digi4LivE, 2024).

This review synthesizes global research on laying hen rearing systems, examining their impact on productivity, welfare, and

sustainability. It aims to provide a comprehensive comparison that can inform both policy-makers and producers in adopting practices that support ethical, efficient, and environmentally responsible egg production.

MATERIALS AND METHODS

This review draws upon a substantial base of validated research published between 2000 and 2024, focusing on studies that examine the relationship between housing systems and key performance outcomes in laying hen production. The analysis encompasses five primary rearing systems: conventional cages, enriched cages, barn systems, free-range systems, and organic systems.

The selected literature includes empirical research addressing egg production, feed conversion efficiency, behavior-based welfare indicators, disease prevalence, mortality rates, consumer preferences, and environmental impact. Additionally, the review identifies management practices and technological innovations that contribute to enhancing animal welfare and improving the overall sustainability of each housing system.

RESULTS AND DISCUSSIONS

Conventional cage systems

Although conventional battery cages have been banned in the EU (European Commission, 1999), they remain prevalent in many regions due to their cost-effectiveness and production consistency. These systems offer controlled environments that reduce disease spread and support efficient feeding and waste management.

Solutions and practices:

- Use of automated climate and lighting control systems enhances production uniformity by stabilizing internal conditions, which supports hen health and consistent egg laying (Tactacan et al., 2009);
- Targeted nutritional interventions (e.g., acidifiers and probiotics) have been shown to support gut health and reduce dependency on antibiotics in high-density environments (Tactacan et al., 2009);
- Beak trimming and aggressive bird monitoring, while controversial, are widely applied to reduce feather pecking due to behavioral restriction, although their use is being replaced by alternative enrichments in progressive systems (Gentle & McKeegan, 2007).



Figure 1. Conventional cages (Source: https://www.bigdutchmanusa.com)

Enriched (furnished) cage systems

Enriched cages were developed to provide behavioral opportunities that conventional cages lack. They include perches, nest boxes, and scratching areas, which significantly improve welfare while retaining much of the productivity of cage systems (Lay et al., 2011).

Solutions and practices:

- Design optimization, such as adjustable perches and partitions, improves spatial distribution and reduces competition among hens, resulting in fewer injuries and stress behaviors (Tauson, 2005);
- Use of red lighting or dimmable light gradients supports circadian regulation and reduces feather pecking, as hens are more relaxed under lower light intensity (Shimmura et al., 2007);



Figure 2. Enriched cages (Source: https://www.bigdutchmanusa.com)

- Sloped flooring with manure belts reduces contact with waste, improving hygiene and egg cleanliness while lowering ammonia emissions (Tauson, 2005).

Barn (floor-based) systems

Barn systems house hens indoors on litter floors, often in single or multi-tier aviary designs, and offer more behavioral freedom. However, they require careful management of density, litter quality, and air exchange to prevent disease and aggression.

Solutions and practices:

- Installation of multi-level aviary structures increases usable space and reduces crowding. This improves access to feed and water while allowing hens to escape aggressive interactions (Rodenburg et al., 2008);
- Litter management strategies, such as regular turning and supplementation with dry organic materials, prevent wet litter conditions that cause footpad dermatitis and respiratory issues (Bestman & Wagenaar, 2003);
- Automated nest box systems with sensor-driven egg collection reduce broken or dirty eggs and ensure more hygienic conditions for both hens and products (Elson, 2004).



Figure 3. Floor based system (Source: https://www.hightoppoultry.com/slatted-floor-system/)

Free-range systems

Free-range systems combine indoor shelter with daytime outdoor access, enabling hens to engage in foraging, dustbathing, and sunbathing. These systems are valued for ethical production but must be managed carefully to balance welfare with biosecurity.

Solutions and practices:

- Rotational paddock systems reduce overgrazing and parasitic contamination of soil, while promoting vegetation recovery and biodiversity (Singh et al., 2017);
- Natural cover (trees, shrubs, shelters) encourages more uniform outdoor area use, reduces stress, and lowers mortality from predator attacks (Elson, 2004);
- Training hens from a young age to use outdoor areas increases range utilization and decreases fear responses, improving welfare outcomes long-term (Gilani et al., 2014).



Figure 4. Free-Range System (Source: https://au.pinterest.com/pin/269723465161493996/)

Organic systems

Organic systems meet the most rigorous standards, requiring certified organic feed, low stocking densities, and no routine antibiotics. These systems support environmental and ethical values but must overcome biological and economic challenges.

Solutions and practices:

- Use of dual-purpose or heritage breeds, better adapted to outdoor conditions, reduces mortality and feather pecking while maintaining acceptable egg production (Kijlstra & Eijck, 2006);
- Agroecological integration, such as on-site composting and closed nutrient loops, increases resource efficiency and minimizes environmental impact (Bracke et al., 2006);
- Proactive disease prevention, including vaccination and herbal supplements, compensates for the restriction on antibiotic use and helps control intestinal parasites and respiratory diseases (van de Weerd et al., 2009).

Assessing the effectiveness of various rearing systems for laying hens requires a comprehensive understanding of the inherent trade-offs among three critical pillars of poultry

production: productivity, animal welfare, and environmental sustainability. Each system presents distinct advantages and challenges, and their performance must be contextualized within both biological and socio-economic frameworks.



Figure 5. Hens under bushes creating a fresh microclimate (Rana et al., 2022)

From intensive indoor housing to extensive organic systems, the type of environment in which laying hens are reared directly influences not only their physical health and behavioral expression but also farm-level outputs such as egg yield, feed conversion efficiency, mortality rates, and resource use. These systems also differ in their capacity to address consumer expectations for ethically produced food, the mitigation of environmental impacts (e.g., greenhouse gas emissions, land and water use), and compliance with evolving animal welfare legislation.

This section draws on a diverse body of empirical research to compare five primary rearing systems: conventional cages, enriched cages, barn systems, free-range systems, and organic systems. It explores how each system performs in relation to production efficiency, welfare outcomes, ecological impact, and market viability. In doing so, the analysis aims to identify best practices and system-specific innovations that could inform more sustainable and ethically sound models of egg production.

Productivity outcomes

Cage systems, particularly in intensive operations, typically show the highest laying rates due to environmental control, feed efficiency, and minimal energy expenditure by hens. Studies have shown egg production exceeding 300 eggs/hen/year under optimal conditions (Petek et al., 2014). However, such high productivity often comes at the cost of behavioral suppression.

Enriched cages, while providing moderate behavioral outlets, maintain comparable productivity. According to Muir et al. (2016), the inclusion of enrichments did not significantly affect laying rates or feed conversion, suggesting that minor welfare upgrades can be achieved without economic penalty.

In barn systems, productivity is slightly reduced due to factors like increased physical activity and social stress. However, when flock size and tier design are managed well, laying rates remain commercially viable (Nicol et al., 2017). Mortality can be minimized through the use of disease-resistant genetic lines and structured flock management.

Free-range systems show variable productivity depending on outdoor conditions and the breed used. Although laying rates can decrease due to foraging activity, studies such as Anderson (2016) suggest that adequate training and optimized range design can close this performance gap.

Organic systems generally have the lowest productivity, influenced by restrictions on synthetic feed additives and medications. Nonetheless, demand for such systems continues to grow, driven by consumer interest in animal welfare and ecological farming (Tauson et al., 2012).

While free-range and organic systems may underperform in terms of laying rates, they have been shown to produce eggs with improved nutritional quality. For instance, Nistor (Cotfas) et al. (2015) found that eggs from free-range systems had higher albumen protein and yolk lipid content compared to those from battery cages. Differences in fatty acid profiles among poultry species also support the nutritional value of eggs from alternative systems, as shown by Usturoi et al. (2021). However, the study also reported a higher microbial load in free-range eggs, highlighting the importance of stringent biosecurity and hygiene measures in alternative systems. Proper storage is essential to preserve egg quality, as shown by Gavril et al. (2013).

Romanian research conducted under real production conditions also supports the viability of alternative systems. For instance, Usturoi et al. (2022) reported that ISA Brown hens reared in aviary lofts achieved comparable productivity and laying intensity to those raised in improved batteries, while showing better health indicators and favorable feed conversion rates. This suggests that, with appropriate management, alternative housing systems can match or even exceed conventional benchmarks in certain parameters.

Welfare assessment

Welfare concerns are most pronounced in conventional cages, where restricted space limits locomotion, perching, and nesting. Chronic stress and high rates of osteoporosis are well-documented in such systems (Riber et al., 2018).

Enriched cages offer moderate improvements. Though space remains limited, provisions like nest boxes and perches enable some expression of species-specific behaviors, reducing aggression and fearfulness (Sherwin et al., 2010).

Barn systems allow for more complex behaviors but increase risks of injuries, feather pecking, and cannibalism. Welfare outcomes improve significantly when environmental enrichment is provided - such as pecking blocks and elevated platforms (de Haas et al., 2014). Managing light intensity and flock composition further contributes to positive welfare outcomes.

In free-range systems, outdoor access improves physical fitness and reduces stereotypies. However, welfare depends heavily on range design and predator protection. Fanatico et al. (2013) reported that shelters and vegetation significantly reduce stress and fear-related behaviors in free-ranging hens.

Organic production is typically associated with the highest welfare standards, due to combined indoor enrichment and pasture access. However, the ban on routine antibiotics requires excellent disease prevention and early detection strategies (Sossidou et al., 2011).

Environmental sustainability

From a resource efficiency standpoint, cage systems have the lowest environmental footprint per egg produced. However, they concentrate manure and emissions, leading to localized pollution (Leinonen et al., 2012).

Alternative systems - barn, free-range, and organic - demand more land and energy per unit of output but provide environmental benefits through better manure distribution and soil health. For example, free-range systems can sequester carbon if vegetation is maintained and stocking density is managed (Singh et al., 2011).

Organic systems integrate ecological practices like on-farm feed production, composting, and rotational grazing, which reduce dependency on external inputs. However, the variability in feed conversion efficiency and outdoor exposure can increase the carbon footprint if not offset by holistic management (Sossidou et al., 2011).

Market trends and policy directions

Growing consumer concern about animal welfare has spurred demand for cage-free and organic eggs. According to Van Horne & Bondt (2013), retail and legislative momentum - especially in the EU - has led to a steady decline in cage-based systems, with enriched cages now serving as a transitional option.

Global certification schemes such as Animal Welfare Approved and EU Organic play a pivotal role in standardizing production practices and informing consumer choice (Blatchford et al., 2016). Government subsidies and green investment funds are essential for helping producers transition to higher-welfare models without incurring economic losses.

CONCLUSIONS

The comparative analysis of laying hen rearing systems highlights the complexity of achieving a simultaneous balance between productive efficiency, animal well-being, and environmental responsibility. This review confirms that each housing system - whether intensive or extensive - offers specific benefits and limitations and thus must be evaluated through a multidimensional lens that includes biological outcomes, ethical considerations, and sustainability goals.

Conventional cages continue to deliver superior productivity and feed efficiency, yet their

incompatibility with current welfare expectations increasingly limits their acceptability. Enriched cages serve as an intermediary solution, offering slight welfare improvements without drastically compromising production. Meanwhile, barn and free-range systems support a broader range of natural behaviors, though they require careful environmental and behavioral management to control health risks and optimize performance. Organic systems, while often preferred from a welfare and consumer perception standpoint, remain the least productive and most resource-intensive.

Technological advancements - such as automated monitoring tools, smart feeding systems, and data-driven health assessments - are emerging as critical tools across all systems, helping bridge the gap between productivity and welfare. Additionally, genetic selection tailored to specific housing types and better environmental design can further support system-specific optimization.

Ultimately, a universal solution does not exist. Progress in egg production must be guided by integrated, science-based strategies that take into account regional legislation, market trends, and societal values. By promoting collaborative efforts among producers, researchers, and policymakers, the poultry sector can evolve toward systems that are not only economically sustainable but also ethically and ecologically responsible.

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THE USE OF "CHLORAMICOB" BIOSTIMULATOR IN THE FEEDING OF NURSE BEES TO OBTAIN ROYAL JELLY

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Abstract

Royal jelly is a secretion of the hypopharyngeal and mandibular glands of young nurse bees, used to feed larvae in the first three days throughout the larval period. The research evaluated the impact of the biostimulator "Chloramicob" on royal jelly production. It was established that the optimal dose is 2.25 ml/L administered to nurse bees (1 L sugar syrup/day for three days). In the first stage, it increased the larval acceptance rate by 12.5-27.5%, the mass of the brood by 2.4-14.4% and the total royal jelly production by 25.45-40.04%. In the second stage, increases of 10.0-24.3% in the number of larvae raised, 1.49-1.79% in the diameter of the queen cells and 41.14-93.38% in total royal jelly production. In the third stage, there were increases of 1.12-1.32% in the diameter of the queen cells, 1.13-3.30% in their length and 4.82-27.82% in the total royal jelly production obtained compared to the control group. The conclusion highlights that the use of the biostimulator "Chloramicob" in the feeding of nurse bees leads to an increase in the number of larvae accepted by 7.27-16.36% and in the total royal jelly production by 21.11-48.06% compared to the control group.

Key words: *bee families, biostimulator, sugar syrup, morpho productive indices, royal jelly.*

INTRODUCTION

Royal jelly is a secretion of the hypopharyngeal and mandibular glands of young bees (nurses) at the age of 3-12 days, which is used to feed young larvae in the first three days, queen larvae throughout the larval period and queens (Ahmad et al., 2020; Ebubekir & Çağlayan, 2023; Eremia, 2020).

Freshly harvested royal jelly presents an opaque mass, consistently like cream, slightly viscous, white, yellowish-white or yellowish in color, sour taste with a burning shade and a specific pleasant smell, slightly aromatic (Eremia et al., 2000).

Royal jelly contains 58.0-67.0% water and 33.0-42.0% dry matter, of which: 13.8-18.0% protein, 7.5-12.5% carbohydrates, 3.0-6.0% lipids, 0.8-1.5% ash, 8.7-4% undetermined substances (Lazăr & Vornicu, 2007).

To produce royal jelly, the nurse colony must have at least 3 kg of bees of all ages and a lot of broods, 10-12 kg of honey and considerable reserves of fodder (Krasochko & Eremia, 2022). The amount of royal jelly harvested depends on the number of larvae proposed for rearing, as well as the number accepted by the nurse colony (Ebubekir & Çağlayan, 2023).

Feeding the brood colonies with the nutritional composition containing sugar syrup and peppermint essential oil makes it possible to increase the percentage of larvae reception, increases the productivity of bee colonies in the production of royal jelly, improves the physiological state of worker bees and stimulates the flight activity when collecting both with and without pollen balls (Krivtsov & Lebedev, 1993).

The process of obtaining royal jelly includes forming brood colonies, making combs and transferring larvae; after 72 hours, the royal jelly is extracted (https://revista-ferma.ro/7-conditii-pentru-obtinerea-laptisorului-de-matca/).

During periods of lack of collection, it is mandatory that the production of royal jelly be stimulated by artificial feeding with syrup (https://agromedia.md/agricultura-moderna/zootehnie/cresterea-albinelor/cum-se-produce-laptisorul-de-matca-procedeul-pas-cu-pas) or with food rich in proteins

(https://apilink.ro/utile/produse-apicole/laptisor-de-matca/59-producerea-laptisorului-de-matca).
Based on the above, the purpose of the work is to study the influence of stimulating nurse bees feeding with a mixture of sugar syrup and a biostimulator on the number of larvae accepted for rearing and the production of royal jelly.

MATERIALS AND METHODS

For the experiment, four groups of bee colonies we had formed, according to the principles of the methods of analogs according to the number of combs, the strength of the colony, the number of combs with brood capped and the honey reserve in the hive.
In the absence of maintenance honey collection, the bee colonies were administered one liter of a mixture of sugar syrup in a concentration of 50% and the biostimulator "Chloramicob" group I - with 1.25 ml/L, group II - with 2.25 ml/L, group III - with 3.25 ml/L, group IV (control) pure sugar syrup.
In the first half, a frame with 40 transferred larvae was introduced into the nurse (breeding) families, in the second half – with 70 larvae (2 frames: one with 30 and the second with 40 larvae) and in the third – with 110 larvae (3 frames: two with 40 larvae and the third with 30 transferred larvae).
The nurse bees were given one liter of a mixture of sugar syrup in a concentration of 50% and with biostimulator, three days in a row after the introduction of the frame with transferred larvae, after 3 days the frames with the bees were removed from the hive to collect royal jelly and the second and third half were repeated.
During the royal jelly collection experiments, the following indices were studied: the percentage of acceptance of transferred larvae for growth, the diameter, length and mass of the brood, the amount of royal jelly obtained from a brood and from a nurse family per session. The results obtained were processed by the method of statistical variations using the computer program Microsoft Office.

RESULTS AND DISCUSSIONS

The experimental bee colonies had 12-13 combs, the strength of the colonies was 10 alleys (spaces between the combs occupied by bees), the honey reserve was 9.0-9.5 kg and 6.0 combs with brood. In the first half, the nurse colonies were given a frame with 40 transferred larvae for rearing.
The results of the research showed that feeding bee colonies during the period of lack of honey collection with a mixture of sugar syrup with a concentration of 50% and biostimulator stimulates an increase in the percentage of larvae acceptance for rearing by 12.5-27.5% compared to the control group (Table 1).

Table 1. Productive indices of breeding families, number of larvae transferred and accepted for breeding, 03-06.06.2024 (I transfer)

Group	No. of frames, pcs.	Colony strength, No. of alleys	Honey reserves in the nest, kg	Amount of capped brood, hundred cells	No. of transferred larvae, pcs.	No. of accepted larvae	
						Pcs.	%
I-Sugar syrup + Chloramicob, 1.25 ml/l	12	10	9.0	6	40	32	80.0
II-Sugar syrup + Chloramicob, 2.25 ml/l	13	10	9.5	6	40	30	75
III-Sugar syrup + Chloramicob, 3.25 ml/l	12	10	9.0	6	40	36	90
IV-Pure sugar syrup (control)	12	10	9.0	6	40	25	62.5

The diameter of the queen cells on the third day in the experimental groups was on average 10.32-10.63 mm and the length – 23.91-25.90 mm. The mass of the queen cells with larvae and royal jelly in group II was 0.18 g (14.4%) higher than in the control group (*$B_3 \geq 0.999$), and in group I - by 0.03 g (2.4%). The largest amount of royal jelly in one queen cell was obtained in group II on average - 0.587 g, which was 84 mg (16.70%) more than in the control group IV, the difference being authentic **$B_3 \geq 0.999$ (Table 2).

The coefficient variation of the studied indices ranged between 5.24 (queen cell diameter) and 13.05 (amount of royal jelly obtained from a queen cell). The amount of royal jelly obtained from one beehive was 1.8-2.3 times higher than the results obtained by other local researchers (Belioglo, 2001).

Considering the number of larvae accepted and the average amount of royal jelly from a queen cell, it was found that the total amount of royal jelly obtained in the first half varied between 15.776 g and 17.610 g, or 3.201-5.035 g (25.45-40.04%) more than in the control group.

It was revealed that the largest amount of royal jelly (17.610 g) was obtained in group II, which was administered a mixture of sugar syrup and 2.25 ml/l of biostimulator. The results obtained confirm the deductions of some Romanian researchers, such as (Mărghitaș, 2008) that from 5 bee colonies, an average of about 50 g of royal jelly can be obtained in one session. Feeding nurse bees during the larval growth period for three days with one liter of a mixture of sugar syrup and 2.25 ml/l of biostimulator increases the production of royal jelly by 40.04% more than in the control group.

In the second half, two frames (one with 40 larvae, the second with 30 larvae) were introduced into the breeding families, a total of 70 transferred larvae. Nurse bees were fed from the introduction of the frames with transferred larvae with one liter of a mixture of sugar syrup with biostimulator for three days until the frame with the beehives was removed from the hive to collect the royal jelly.

It was found that the breeding families in the experimental groups in the second half where 70 transferred larvae were given accepted 28-38 larvae or 10.0-24.3% more than the control group IV (Table 3).

Table 2. Indices of the brood and the amount of royal jelly obtained, 03-06.06.2024 (I transfer - 40 larvae)

Group	Indices	Queen cell's diameter, mm	Queen cell's lenght, mm	The queen cell's mass with larvae and royal jelly, g	The amount of royal jelly obtained from a queen cell, g	The total amount of royal jelly, g
I - Sugar syrup + Chloramicob, 1.25 ml/l	$\bar{X}\pm S_{\bar{x}}$	10.32±0.103	23.91±0.415	1.28±0.018	0.493±0.008	15.776
	V, %	5.66	9.83	7.99	9.79	-
II - Sugar syrup + Chloramicob, 2.25 ml/l	$\bar{X}\pm S_{\bar{x}}$	10.41±0.105	25.90±0.425	1.43±0.031*	0.587±0.012**	17.610
	V, %	5.24	8.98	11.80	11.25	-
III - Sugar syrup + Chloramicob, 3.25 ml/l	$\bar{X}\pm S_{\bar{x}}$	10.63±0.096	23.94±0.369	1.21±0.021	0.454±0.010	16.344
	V, %	5.43	9.24	10.62	13.05	-
IV - Pure sugar syrup (control)	$\bar{X}\pm S_{\bar{x}}$	10.48±0.125	25.04±0.268	1.25±0.021	0.503±0.010	12.575
	V, %	5.95	5.35	8.45	9.78	-

*Mass of the queen cell with larva and royal jelly: group II / group IV – $*B_3 \geq 0.999$
**Quantity of royal jelly obtained from a queen cell: group II / group IV – $**B_3 \geq 0.999$.

Table 3. Number of larvae transferred and accepted for rearing, 06-09.06.2024 (II transfer)

Group	No. of transferred larvae, pcs.	No. of accepted larvae	
		pcs.	%
I - Sugar syrup + Chloramicob, 1.25 ml/l	70	28	40.0
II - Sugar syrup + Cloramicob, 2,25 ml/l	70	38	54.3
III - Sugar syrup + Chloramicob, 3.25 ml/l	70	34	48.6
IV-Pure sugar syrup (control)	70	21	30.0

It was revealed that the diameter of the queen cells in the experimental groups was on average 10.23-10.26 mm or 0.15-0.18 mm (1.49-1.79%) higher than in the control group IV, the length of the queen cells respectively – 21.41-22.05 mm (2.47-2.94%) (Table 4).

From one queen cell in the experimental groups, an average of 0.413-0.420 g of royal jelly was obtained or 0.020-0.027 g (5.10-6.87%) more than in the control group. The largest amount of royal jelly was obtained from

group II - 15.960 g or 93.38% higher than the control group.
The coefficient of variation of the studied indices ranged between 5.79 (diameter of the queen cell) and 13.41 (amount of royal jelly obtained from one queen cell).
So, in the second half, when 2 frames with 70 larvae (30+40) were raised, and the feeding of nurse bees stimulated the acceptance of larvae by 10.0-24.3%, the growth of the queen cells (diameter - by 1.49-1.79%, length - 2.47-2.80%, mass - 2.86-84.76%) and the amount of royal jelly obtained from one queen cell by 5.09-6.87%, and the total amount - by 41.14-93.38%.
In the third half, three frames were introduced into the experimental colonies (the first and second with 40 larvae each, the third - with 30 larvae), a total of 110 transferred larvae.
The research results demonstrated that in the third round, when a larger number of larvae (110 pcs.) was administered, 41-51 pcs. were accepted in experimental groups, or 2-12 pcs. (1.9-10.9%) more than in the control group (Table 5).

Table 4. Indices of the brood and the amount of royal jelly obtained, 06-09.06.2024
(II transfer - 70 larvae)

Group	Indices	Queen cell's diameter, mm	Queen cell's lenght, mm	The queen cell's weight with larvae and royal jelly, g	The amount of royal jelly obtained from a queen cell, g	The total amount of royal jelly, g
I - Sugar syrup + Chloramicob, 1.25 ml/l	$\bar{X}\pm S\bar{x}$	10.26±0.133	21.41±0.326	1.08±0.024	0.416±0.008	11.648
	V, %	6.86	8.06	11.51	10.16	-
II - Sugar syrup + Chloramicob, 2.25 ml/l	$\bar{X}\pm S\bar{x}$	10.25±0.088	22.05±0.231	1.94±0.017*	0.420±0.009	15.960
	V, %	5.79	6.46	9.73	13.41	-
III - Sugar syrup + Chloramicob, 3.25 ml/l	$\bar{X}\pm S\bar{x}$	10.23±0.129	21.95±0.257	1.09±0.016	0.413±0.009	14.042
	V, %	7.35	6.81	8.49	12.64	-
IV - Pure sugar syrup (control)	$\bar{X}\pm S\bar{x}$	10.08±0.129	21.42±0.386	1.05±0.030	0.393±0.008	8.253
	V, %	5.88	8.25	13.1	9.49	-

*Mass of the queen cell with larva and royal jelly: group II/group IV – *B3 ≥ 0.999

Table 5. Number of larvae transferred and accepted for rearing, 09-12.06.2024
(III transfer)

Group	No. of transferred larvae, pcs.	No. of accepted larvae	
		pcs.	%
I-Sugar syrup + Chloramicob, 1.25 ml/l	110	41	37.3
II-Sugar syrup + Chloramicob, 2,25 ml/l	110	49	44.5
II-Sugar syrup + Chloramicob, 2.25 ml/l	110	51	46.3
IV-Pure sugar syrup (control)	110	39	35.4

The diameter of the queen cells obtained from the experimental groups varied, on average, between 9.97 and 9.99 mm, or 0.11 and 0.13 larger than the control group, the length - 20.14-20.98 mm and the mass of queen cells with larva and royal jelly - 0.87-0.95 g. The coefficient of variation ranged between 6.45 (queen cell length) and 96.58 (amount of royal jelly obtained from a queen cell).
The largest amount of royal jelly from a queen cell was obtained from group II, on average 0.352 g, and the total amount - 17,248 g or 3.754 g more than the control group (Table 6).
Therefore, we can mention that increasing the number of frames (3), the number of larvae (110 pcs.) and stimulating the nurse bees with sugar syrup in a concentration of 50% and with a biostimulator increased the growth of the diameter of the queen cells by 1.12-1.32%, the length - by 1.13-3.30%, the mass of the queen cells with larvae and royal jelly - by 2.20-4.40%, the amount of royal jelly obtained from a queen cell – by 1.73% and the total amount of royal jelly obtained per group by 4.82-25.28% compared to the control group.

In total, over three rounds, the breeding colonies that were introduced with frames (1-3) with 40, 70 and 110 transferred larvae and the feeding of nurse bees with a mixture of sugar syrup and biostimulator in doses of 1.25-3.25 ml/l increased the number of transferred larvae accepted for rearing by 7.27-16.36% and the total amount of royal jelly obtained by 21.11-48.06% more than the control group (Table 7). It was found that the largest amount of royal jelly (50.818 g) over three rounds was collected from bee colonies in group II, which were introduced to grow 1-3 frames with 40-70-110 transferred larvae and nurse bees fed with a mixture of sugar syrup and a 2.25 ml/l biostimulator, one liter each when introducing the frames with larvae and daily for three days until the frames were removed from the hive, then the second and third rounds were repeated similarly.

Table 6. Morphometric indices of the brood and the amount of royal jelly obtained, 09-12.06.2024 (III transfer - 110 larvae)

Group	Indices	Queen cell's diameter, mm	Queen cell's lenght, mm	The queen cell's mass with larvae and royal jelly, g	The amount of royal jelly obtained from a queen cell, g	The total amount of royal jelly, g
I - Sugar syrup + Chloramicob, 1.25 ml/l	$\bar{X}\pm S\bar{x}$	9.97±0.101	20.14±0.305	0.93±0.016	0.345±0.007	14.145
	V, %	6.47	9.80	11.24	12.59	-
II - Sugar syrup + Chloramicob, 2.25 ml/l	$\bar{X}\pm S\bar{x}$	9.99±0.098	20.98±0.195	0.95±0.017	0.352±0.008	17.248
	V, %	6.80	6.45	12.13	11.06	-
III - Sugar syrup + Chloramicob, 3.25 ml/l	$\bar{X}\pm S\bar{x}$	9.98±0.134	20.54±0.337	0.87±0.038	0.308±0.011	15.708
	V, %	9.55	11.25	17.75	24.95	-
IV - Pure sugar syrup (control)	$\bar{X}\pm S\bar{x}$	9.86±0.103	20.31±0.280	0.91±0.020	0.346±0.053	13.494
	V, %	6.54	8.87	13.79	96.58	-

Table 7. Number of transferred larvae and amount of royal jelly obtained during three rounds (03-06; 06-09 and 09-12.06.2024)

Group	No. of larvae transferred at one round/ accepted			The total number of transferred larvae / accepted/%	No. of larvae transferred at one round / the amount of obtained royal jelly g/%			The total amount of obtained royal jelly, g	Difference from the control group	
	40	70	110		40	70	110		G	%
I - Sugar syrup + Chloramicob, 1.25 ml/l	32	28	41	220/101/ 45.91	15.776/ 125.46	11.648/ 141.14	14.145/ 104.82	41.569	7.247	121.1 1
II - Sugar syrup + Chloramicob, 2.25 ml/l	30	38	49	220/117/ 53.18	17.610/ 140.04	15.960/ **193.38**	17.248/ 127.82	50.818	16.49 6	148.0 6
III - Sugar syrup + Chloramicob, 3.25 ml/l	36	34	51	220/121/55. 0	16.344/ 129.97	14.042/ 170.14	15.708/ 116.41	46.094	11.77 2	134.3 0
IV - Pure sugar syrup (control)	25	21	39	220/85/ 38.64	12.575/ 100.0	8.253/ 100.0	13.494/ 100.0	34.322	-	100.0 0

CONCLUSIONS

1. It was evaluated that the optimal dose of the biostimulator "Chloramicob" used in the nutrition of nurse bees is 2.25 ml/L sugar syrup.
2. Feeding nurse bees with a mixture of 50% sugar syrup and 2.25 ml/L of biostimulator, one liter each when introducing frames with 40 transferred larvae and daily for three days until removing the frames from the hive, stimulates an increase in the percentage of larvae acceptance for growth by 12.5-27.5%, the mass of queen cells with larvae and royal jelly by 2.4-14.4% and the total amount of royal jelly

obtained by 25.46-40.04% higher than the control group.

3. In the second half, when introducing 2 frames with 70 (30+40) larvae transferred after feeding the nurse bees, it was revealed that in the experimental groups the number of larvae increased by 10.0-24.3%, the diameter of the queen cells by 1.49-1.79%, the amount of royal jelly obtained from a queen cell by 5.10-6.87%, the total amount of royal jelly obtained in the second half by 41.14-93.38% higher than in the control group.

4. In the third round, the introduction of three frames with 110 (30+40+40) transferred larvae increased the growth of the diameter of the queen cells by 1.12-1.32%, the length - by 1.13-3.30%, the mass of queen cells with larvae and royal jelly - by 2.20-4.40%, the amount of royal jelly obtained from one queen cell - by 1.73% and the total amount of royal jelly obtained per group by 4.82-27.82% compared to the control group.

5. It was found that the use of the biostimulator in the nutrition of nurse bees during three feedings ensures an increase in the number of transferred larvae accepted for growth by 7.27-16.36% and the total amount of royal jelly obtained by 21.11-48.06% higher than the control group.

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ANALYSIS OF MILK PRODUCTION AND MILK QUALITY IN MONTBELIARD COWS FROM A FARM IN SOUTHEASTERN ROMANIA

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Abstract

Milk has had a fundamental importance in nutrition over the centuries, mainly due to its high nutritional value, which results from a diversified chemical composition, including fat, protein, lactose, and mineral content. This study aims to monitor milk production and the evolution of the main milk quality parameters such as fat, protein, lactose, dry matter, and somatic cell count. The study was carried out on a farm in southeastern Romania, on Montbeliard cows raised in an intensive system. The study was conducted over 12 months, covering both the warm and cold seasons. The results highlighted the importance of monitoring milk production and that a well-balanced nutrition, which includes high-quality feed, is essential for maximizing milk yield, but also for its chemical composition.

Key words*: cow farm, dairy cows, fat content, milk quality, protein content.*

INTRODUCTION

Milk is generally defined as a whitish liquid secreted by the mammary glands of female mammals (Ben Franj et al., 2023). Milk has played a fundamental role in nutrition over the centuries, mainly due to its high nutritional value, which results from a diversified chemical composition, including fat, protein, lactose, and mineral content (Dror & Allen, 2014; Mihai et al., 2019; Kapaj & Kapaj, 2021). Notably, approximately 87.7% of milk is water, with the remaining constituents distributed in various forms, and the proportion of each component differs between breeds (Tyasi et al., 2015). Feeding management practices on dairy farms significantly influence the levels of fat and protein concentration in milk. Consequently, all factors affecting milk composition including nutrition, and feeding management have the potential to compromise milk quality (Tyasi et al., 2015). The quality and composition of milk can vary significantly depending on a number of factors such as breed, diet, season, stage of lactation, husbandry system, environmental conditions, etc. (Ben Franj et al., 2023; Kostovoska et al.,2024).

In Romania, the milk and dairy sector constitutes one of the most significant segments of national agriculture, representing more than 25% of total agricultural production and approximately 10% of animal production. The main source of milk comes from the exploitation of animals in the private sector, which contributes over 95% to total milk production (Porosnicu et al., 2020). Milk quality is one of the major challenges in Romanian cow farms, especially regarding parameters influenced by hygiene (Răducu C. et al., 2016).

Among the most widespread breeds of cows in Romania are Holstein, Montbeliard, Romanian Spotted (in Romanian: Bălțată Românească), Brown (in Romanian: Brună) and Jersey (Grăsinaru et al., 2018). The Montbéliarde cattle breed became known in Romania in 2009, after importing several animals from France. Today, Montbéliarde cattle are raised in several countries on five continents, and they are known globally for their dual purpose of milk and meat production (Mirulugovna, 2020; Soatov et al., 2021). Although primarily raised for milk production, Montbéliarde also demonstrates significant potential in the production of quality meat. The breed does not require very special care, for this reason, it has successfully adapted to the environmental conditions of the area, Romania having a temperate continental climate (Mihai et al., 2019).

The main purpose of this paper is to analyze milk production from Montbeliard cows, and the evolution of the main milk quality parameters such as fat, protein, lactose, dry matter, and somatic cell count.

MATERIALS AND METHODS

The study was conducted over 12 months between January and December 2024, at a Montbeliard cow farm in southeastern Romania to monitor milk production and determine chemical composition and somatic cells. The research included a total of 350 Montbeliard cows raised on an intensive farm. All animals included in the study were selected with great care, taking into account similarity criteria in terms of weight, age, and health status. The methods for assessing milk quality consisted of physicochemical and microbiological analyses. Milk samples were taken daily for laboratory analyses, such as determining the percentage of fat, protein, and lactose, using an automatic milk analyzer according to ISO 9622:2013. Each sample was obtained through standardized procedures, using sterile containers to avoid contamination and, if necessary, a preservative such as bronopol. The samples were collected in sterile containers, labeled and placed in an ice box, and transported immediately to the laboratory. The samples were collected in the morning at the first milking and their analysis was performed within two to three hours after sampling.

Located in southeastern Romania, Călărași County is characterized by a dominant relief of plains and meadows, with an average altitude of 46 meters, with maximums of up to 83 meters and minimums of 8 meters. The climate is temperate-continental, influenced by the uniformity of the plain relief, manifested by very hot summers and cold winters. Located on the course of the Danube and the Borcea branch, the region occupies a significant portion of the Muntenia Plain.

For statistical analysis, the data obtained were analyzed using the ANOVA and FISHER tests.

For statistical analysis, the following intervals of the variables were considered: fat ≥ 3 and $\leq 4\%$; protein ≥ 3 and $\leq 4\%$; lactose ≥ 4.5 and $\leq 5.5\%$. All parameters were evaluated using an analysis of variance (ANOVA), analyzing the variations in the chemical composition of milk and milk production over the 12 months. For all statistical analyses, $p < 0.05$ had a significant effect, and at $0.05 < p \leq 0.1$ there was a trend.

RESULTS AND DISCUSSIONS

Farm description

The farm studied is divided according to Table 1. The stables are made of metal structures with large openings (large air volume), with a height of 5 m at the eaves and 11 m at the ridge. The livestock farm is composed of 4 stables (3 for lactating cows and a stable for calves between 0 and 6 months - maternity). For raising dairy cows, the farm's activity is focused on ensuring the comfort and welfare conditions of the animals in the 4 shelters in order to obtain better results regarding the quantity and quality of the finished product.

Table 1. Shelter size and farm capacity in Călărași County

Cowshed	Area (m^2)	Number max
Cowshed 1	3200	150 lactating cows
Cowshed 2	3200	150 lactating cows
Cowshed 3	2820	120 cows in maternity
Cowshed 4	2800	120 lactating cows
Total	12.020	

Description of the technological flow

The interior of a stable was divided into three distinct areas: a) the feeding area consists of two perimeter alleys (Figure 1) where the animals receive food. The alleys are sufficiently wide for the machines that administer the feed to access them. Access to the feed is through a specially arranged, self-capturing fence, with feeding possible for each animal; b) the animal rest area is provided with individual boxes separated by a metal bar. These boxes offer increased comfort to the animals, who are on chopped straw bedding provided with dividing bars. Each animal has its own resting place and an adjustable tube depending on its size, but it is not possible for the animal to defecate on the resting bed; c) the free area where the animals move and have access to the feeding, watering, and resting areas. The animals are watered using tilting stainless steel drinkers with a constant level and water flow depending on the number of animals in the respective batch. Access to water is

permanent, allowing the animal to be watered as often as needed.
The animal circulation alley is made of rolled concrete with a slight slope with an inclination towards the scraper bridge gutter that pushes the manure at the end of the stable into a concrete collector channel and a concrete collection pit, respectively, allowing the manure to be stored. The evacuation of manure from the movement alley is done using the scraper plow.

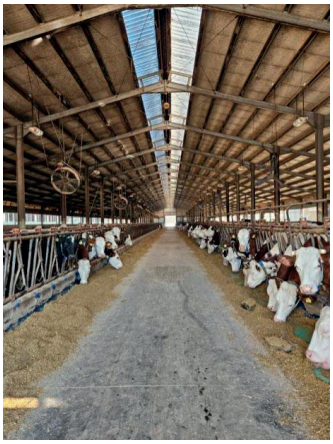


Figure 1. Feeding area consisting of two alleys, in a dairy farm (original)

The milking process

The animals are directed to the milking parlor (area 200 m^2) (Figure 2) in production batches, avoiding crowding and animal stress.

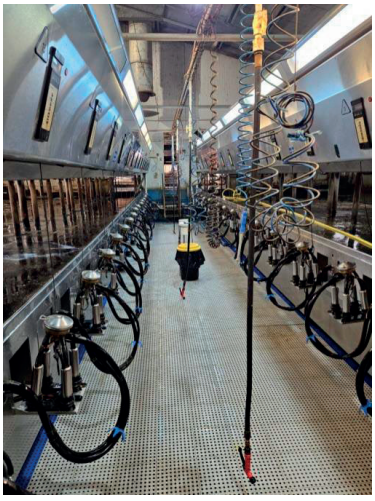


Figure 2. Cow milking area automatic milking system (original)

The duration of milking for an animal is 12 minutes, after which the animals return to the shelter through specially arranged corridors, equipped with gates (closing-opening), so that they reach the stable area to be fed. The milk milked from the animals is pushed with a pump on a stainless steel pipe to the cooling and storage tank, which keeps the milk at a temperature of 4°C, from where the milk is delivered.

Microclimate

The cattle shelter ensures an appropriate microclimate in terms of the construction itself, which is made of thermally insulating materials with access routes for feed administration and manure evacuation, drainage channels and a paved resting area. The brightness of the shelters is ensured by spaces with vertical openings with an area of 1/20 of the total area of the shelter. The bedding is changed daily, keeping it dry to prevent manure from depositing, especially on the back of the cow (udder and other nearby portions) and to avoid infections.

Feeding

Other spaces within the zootechnical complex are used to store fibrous feed and silage corn. The animals are fed with feed from their own production (Figure 3).



Figure 3. Feeding dairy cows with stock feed (original)

For the preparation of concentrated feed, there is a mill on the farm premises for grinding and mixing appropriate feeding recipes. Throughout the 12 months of the study, the cows' nutrition

was not modified; they were fed with stock feed.The cows' ration consists of fully ground corn on the cob, corn silage, wheat bran, barley, oats, soybean meal, alfalfa hay, semi-hay and sphagnum.

Milk composition

Table 2 presents a summary of three components of cow's milk: fat, protein and lactose, evaluating them by means of the mean, standard deviation, and minimum and maximum values. The mean fat content is 3.62%, which indicates a relatively constant concentration in most of the samples analyzed.

Table 2. Milk composition: Mean, Standard Deviation, Minimum and Maximum for fat, protein and lactose

	Fat content	Protein content	Lactose content
Mean	3.62	3.57	4.83
Std. Deviation	0.1	0.08	0.05
Minimum	3.51	3.46	4.73
Maximum	3.8	3.67	4.92

The standard deviation of 0.1 suggests a low variability around this mean. The minimum and maximum values, of 3.51% and 3.8%, respectively, show that fat levels are well controlled and do not undergo significant variations over the 12 months. For protein, the mean is 3.57%. The standard deviation of 0.08 also indicates a low variability. The minimum and maximum values, of 3.46% and 3.67%, respectively, reflect the lack of significant differences over the 12 months, the total difference being 0.21%. As for lactose, the mean is 4.83%. The standard deviation of 0.05 suggests an extremely low variability, indicating a pronounced uniformity of this component in the analyzed samples. The minimum and maximum values, of 4.73% and 4.92%, denote a similar consistency inlactose content, without significant differences. The data in Table 2 suggest that the investigated samples have a stable content, with minimal variations in fat, protein and lactose levels.

Figure 4 provides information on the fat, protein and lactose content of cow's milk, analyzed during each month of 2024. Regarding fat, a slight variation is observed, with the highest values in October (3.80%) and the lowest in April (3.51%). This suggests a general trend of increasing fat content in the autumn season, which could be influenced by the microclimate or the health of the cows. It is worth noting that the nutrition of the cows was not changed throughout the analysis. The protein content remains relatively constant throughout the year, with minimum values observed in June (3.46%) and maximum values in October, November and December (3.67%). This stability suggests uniformity in the nutrition and health of the animals, with no significant fluctuations affecting the quality of the milk proteins. Regarding lactose, the content fluctuates insignificantly throughout the year, with the lowest values in January (4.73%) and the highest in April (4.92%). These minimal variations indicate possible seasonal influences, suggesting that the composition of milk is affected by environmental conditions, especially during winter and summer.

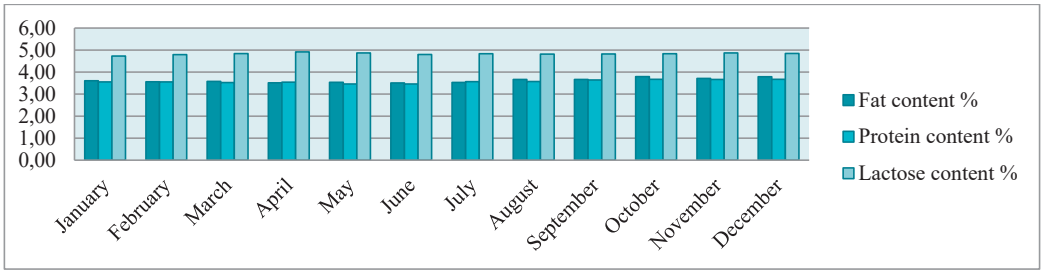


Figure 4. Variation in protein, fat and lactose content over 12 months

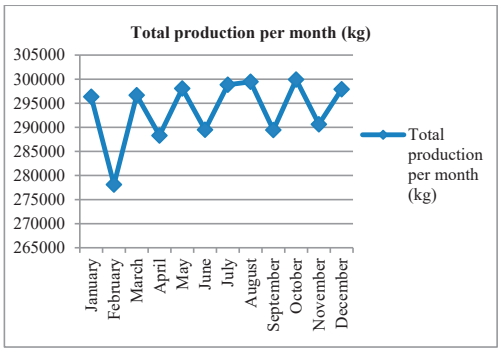


Figure 5. Total milk production per month for 12 calendar months

The analysis of total milk production throughout the year highlights significant variations throughout the period, with a peak recorded in October when production reached 299,925 kg (Figure 5). This is possibly influenced by favorable climatic conditions and maintenance technology. In contrast, February recorded the lowest production, with 278,110 kg, which may indicate possible challenges encountered during the winter period, such as animal welfare, but also the fact that February has fewer days than the others. Most months in the analyzed interval show a relatively constant production, with values fluctuating around the average. For example, milk production in January (296,360 kg) and March (296,670 kg) is similar, which suggests stability in feed management and cow health. August saw a notable increase in production (299,460 kg), indicating increased efficiency in animal care and feeding practices. Overall, the data suggests a positive trend in total milk production.

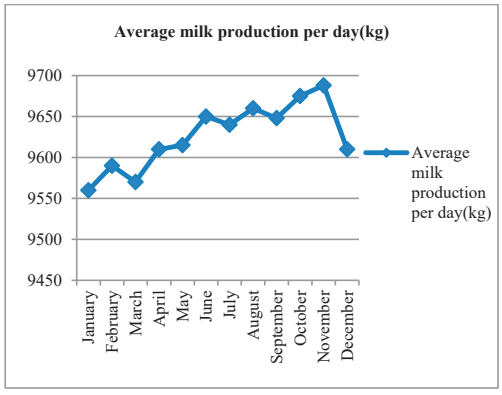


Figure 6. Average milk production per day (kg)

Analysis of the average daily milk production, as shown in Figure 6 with the data presented, highlights a general trend of slight increase throughout the year, with minimal variation between months. Starting with January, daily production was 9,560 kg, and in December a value of 9,610 kg was recorded, suggesting stability on the farms throughout the year. This analysis suggests that farmers had to continue to optimize their care practices to maintain these production levels. Overall, the data reflects a good performance of the dairy herd with minimal variations.

CONCLUSIONS

The analysis of the chemical composition of the milk showed a slight variation in fat content, with maximum values in October (3.80%) and minimum values in April (3.51%). The protein content remained relatively constant throughout the year, with minimum values in June (3.46%) and maximum values in October, November and December (3.67%). The lactose content showed insignificant fluctuations, with the lowest values in January (4.73%) and the highest in April (4.92%). The results obtained emphasize that although the nutrition of the cows was not modified during the analysis, the health of the cows, environmental conditions and maintenance technology can influence the chemical composition of the milk.

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ROMANIA'S LIVESTOCK SECTOR: CHALLENGES AND PERSPECTIVES ON PRODUCERS' ASSOCIATIONS

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Abstract

The paper proposes an analysis of producer associations within Romania's zootechnical sector. The research focuses on the structure, distribution, and activities of producer associations, highlighting their importance in rural development and identifying key challenges and opportunities related to the use of European funds. Documentation included the use of open-access articles available on platforms such as Google Scholar, ResearchGate, and Clarivate. Information about producer associations in the livestock sector was sourced from the website of the Ministry of Agriculture and Rural Development, while data on economic agents active in NACE Code 014 - Animal Raising - were obtained from the TopFirme.com platform. Relevant information from the websites of industry-specific associations was also selected to complement the analysis. The collected data were statistically processed and interpreted. The market concentration was evaluated using the Gini-Struck index calculation method. The research results indicate a low number of associative entities at the national level and a low market concentration, thereby highlighting the untapped potential of the zootechnical sector. The development of associative forms in Romania's zootechnical sector must be supported and promoted through public policies and measures for fiscal and administrative assistance.

Key words: *Gini-Struck Index, livestock production, producers' associations, rural development, Romania.*

INTRODUCTION

The zootechnical sector represents a significant component of Romanian agriculture, both in terms of its contribution to GDP and its impact on supporting rural areas. The territory inhabited by the Romanian population has a long-standing tradition of animal husbandry, facilitated by favorable natural resources: pastures with diverse vegetation, flowing water sources, and salt deposits essential for sheep and cattle. Unfortunately, the seasonal nature of production on upland pastures imposes major nutritional constraints on livestock production systems (Ministry of Agriculture and Rural Development - MADR, 2015).

Although animal-based food products are vital for the population and form a key component of national food security, Romania faces challenges in supplying domestically-produced goods to its own consumers, with the domestic market relying heavily on imports. In terms of the number of economic operators, the Romanian zootechnical sector is less developed compared to the crop farming sector.

Research conducted by Ziarul Financiar reveals that the number of companies active in animal husbandry is 2.5 times lower than that of operators involved in cereal cultivation (Nițu, 2023).

Approximately 8,500 companies are registered in Romania for cereal cultivation, while only 3,300 companies declare zootechnical activities, as reported by Termene.ro data (2024).

Romania's zootechnical potential is underutilized, with the local sector characterized by a smaller number of animals relative to the country's agricultural land area compared to Western Europe (AgroTV, 2022). Additionally, Romanian farmers fail to maximize the value of crop production through the zootechnical sector, with much of the harvest being sold as raw material.

Research made by Sandu (2015) highlights a substantial reduction in livestock populations in Romania between 1990 and 2013: cattle (-59.18%), pigs (-66.22%), sheep (-35%), and

poultry (-44%), with only goat populations increasing by around 30%.

Animal husbandry should aim to ensure sufficient livestock numbers to meet the population's consumption needs while improving the genetic value of breeds to boost domestic consumption and export potential.

Vidu (2023), in an interview with the specialized journal Agrointeligenta, mentioned that the development of Romania's livestock sector occurs through two systems: intensive-industrial and traditional. While the intensive method ensures higher productivity, traditional animal husbandry methods should not be overlooked.

Romania's natural pastures have significant potential for livestock rearing, and the national zootechnical sector could theoretically feed over 70 million consumers without relying on imports (Gelatu, 2023).

Ensuring good breeding conditions in livestock farms, in compliance with European animal welfare regulations, is another critical characteristic necessary for the local sector (Enea et al., 2023).

National legislation outlines the following possible forms of association and cooperation in agriculture: producer groups, agricultural companies, associations, and cooperatives. These forms of association are diverse, shaped by the relationships established between agriculture and other sectors of the national economy, as well as within agriculture itself, between economic agents and units for storage, processing, and distribution of agricultural products (Agrointeligenta, 2015).

According to current legislation, associations in Romanian agriculture can take the following forms: agricultural companies and other types of associations in agriculture (Law No. 36/1991, with amendments); associations (Government Ordinance No. 26/2000, approved by Law No. 246/2005, with amendments); and agricultural cooperatives (Law No. 566/2004, with amendments) (Parliament of Romania, 1991).

The development of producer associations can be a source of economic growth, optimizing the use of local resources and capitalizing on regional advantages. Members of agricultural associations can gain additional economic benefits, positively impacting rural communities' welfare. At the European level, the development of specialized agricultural cooperatives in Western states, organized across various levels, is a key factor in increasing farmers' competitiveness, achieving high-performance products, and securing higher prices for cooperative members. Romanian farmers can benefit from Western experience in developing agricultural associations (Gherman et al., 2016).

A characteristic of Romanian agricultural cooperatives is their low level of investment in innovation, promotion, or rural development activities. Local associations, relatively newly established, differ from their Western counterparts, which have a tradition spanning hundreds of years. In Western cooperatives, local production is fully utilized (with activities such as collection, processing, and joint marketing), whereas Romanian units often limit themselves to selling raw materials or conducting imports (Marin & Turek-Rahoveanu, 2015).

A study by Crețu & Tudor (2020) among producer groups in Călărași focused on specific aspects of the development of associative forms in the analyzed area (results achieved, difficulties, advantages, disadvantages, and future plans). The research showed that the main benefits were increased negotiation power with suppliers and buyers, reduced production costs per unit, and the introduction of modern agricultural technologies. The success of associations strongly depends on the cohesion of cooperative members and the level of human involvement.

The agricultural sector is a crucial component of the Romanian economy but is affected by high inflation, rising prices of fertilizers and fuels, and variable climatic conditions (severe drought, floods, extreme temperatures), leading to significant losses for local farmers. Producer associations could mitigate these factors by reducing risks (Florea et al., 2019).

Research by Florea et al. (2020) investigates investment options for managers of 219 agricultural cooperatives in Romania, highlighting the importance of modernizing storage facilities, processing primary products to obtain high-value-added goods,

digitization, and promoting the circular economy.

Results reveal that these investments can strengthen farmers' roles in the food supply chain, reduce specific risks, and ensure sustainable development, with a direct impact on national food security.

Excessive fragmentation of agricultural land in Romania, due to the lack of vision in land restitution policies after the political regime change and the inefficiency of applied reparceling measures, has led to 3.422 million agricultural holdings, the highest number in the EU Member States, over 92% of which have utilized agricultural areas of less than 5 hectares.

A study by Zlati et al. (2023) analyzes the reasons why farmers join agricultural associations, using the fsQCA method. The main negative influence factors identified were governmental policies, which led to the disappearance of several producer groups in the South-East region, emphasizing the importance of state institutional support for the viability of these entities.

MATERIALS AND METHODS

For the bibliographic documentation, representative articles were selected from the Clarivate, Google Scholar, and ResearchGate databases, complemented by publications and journals of producer associations.

Legislative regulations and information on accessing European funds were obtained from documents issued by the European Commission, the Ministry of Agriculture and Rural Development, and the Agency for Financing Rural Investments.

Data regarding producer associations in Romania's zootechnical sector were extracted from the database of the Ministry of Agriculture and Rural Development. For the economic analysis of the livestock sector, statistics from TopFirme.com were used.

The selected information was processed, graphically represented, and interpreted. To evaluate the concentration of associations in specific areas, the method based on the Gini-Struck Index calculation was applied.

The obtained results were compared with findings from related studies in the specialized literature.

RESULTS AND DISCUSSIONS

Key Economic Features of Romania's Livestock Industry

According to "NACE Code 014 - Animal Raising", this group includes the raising and breeding of all animals, except aquatic animals. It excludes activities related to the care and maintenance of farm animals (NACE 0162) and the production of raw hides obtained from the slaughter of animals in slaughterhouses (NACE 1011) (NACE Codes.RO, 2024). The classes within this group include the following activities (Table 1).

Table 1. Specific Activities for NACE 014

NACE Code	Activity
0141	Raising of dairy cattle
0142	Raising of other cattle
0143	Raising of horses and other equines
0144	Raising of camels and camelids
0145	Raising of sheep and goats
0146	Raising of pigs
0147	Raising of poultry
0149	Raising of other animals

Source: Coduri CAEN.RO (2024).

According to data provided by the TopFirme.com platform, there are approximately 3,299 economic operators with their main activity declared under the NACE Code 014 group. The distribution of economic operators across the eight specific activity categories is uneven (Figure 1).

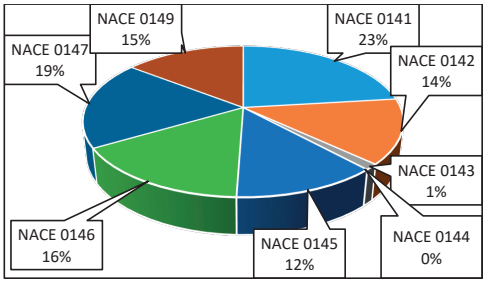


Figure 1. Companie by NACE activities
Source: Authors, by using TopFirme.com (2024)

According to data provided by the TopFirme.com platform, there are approximately 3,299 economic operators with their main activity declared under NACE Code 014 - Animal Raising. The distribution of economic operators across the eight specific activity categories is uneven (Figure 1). Rankings of companies are made by the platform based on the latest submitted financial statements, for informational purposes (TopFirme.com, 2024).

The "NACE Code 0141 - Raising of Dairy Cattle in Romania" presents the following data: 762 economic operators, representing 0.03% of the total economic operators in Romania, with a total turnover of 1.3 billion lei/approximately 299 million euros (equivalent to 0.05% of the country's total turnover), and a net profit of 153.2 million lei/approximately 34.8 million euros (0.03% of the total national net profit). The sector employs 2,831 workers, representing 0.07% of the total employees in Romania. A Top 5 ranking of counties by turnover includes Alba (262.7 million lei/9.7 million euros), Bistrița-Năsăud (96.6 million lei/22 million euros), Mureș (87 million lei/19.8 million euros), Arad (84.2 million lei/19.1 million euros), and Brașov (82.9 million lei/18.8 million euros). The counties of Alba, Bistrița-Năsăud, and Mureș have a strong presence in this sector in terms of both the number of economic operators and turnover. Although the sector represents a relatively small share of the Romanian economy, it plays a key role in ensuring milk and dairy product production, contributing to national food security and rural development.

In the "NACE Code 0142 - Raising of Other Cattle" sector in Romania, 455 economic operators are registered (0.02% of the total economic operators in Romania), with a cumulative turnover of 947.3 million lei/approximately 215.3 million euros (0.04% of the total national turnover) and a net profit of 57.7 million lei/approximately 13.1 million euros (0.01% of the national net profit).

This sector employs 1,062 people, representing 0.03% of the total workforce in Romania. The Top 5 counties by turnover include Maramureș (137.5 million lei/31.3 million euros), Prahova (126.4 million lei/28.7 million euros), Bihor (97.4 million lei/22.1 million euros), Sibiu (94 million lei/21.4 million euros), and Harghita (69.2 million lei/15.7 million euros).

The counties of Maramureș, Prahova, and Bihor have a strong presence in this sector in terms of the number of economic operators and turnover. The sector plays an important role in supplying the domestic market with beef.

40 economic operators are registered with their primary activity under "NACE Code 0143 - Raising of Horses and Other Equines", with a combined turnover of 98.3 million lei/approximately 22.3 million euros, a net profit of 1.8 million lei/approximately 409,730 euros, and 666 jobs. The Top 5 counties include Bucharest (68.7 million lei/15.6 million euros), Bihor (18.3 million lei/4.2 million euros), Arad (4.1 million lei/936,990 euros), Ilfov (1.6 million lei/357,445 euros), and Sibiu (1.3 million lei/293,374 euros). The sector plays an important role in promoting equestrian activities and conserving horse breeds. Bucharest and Bihor cover approximately 85% of the number of economic operators as well as turnover.

A single economic operator, located in Vâlcea County, conducts activities declared under "NACE Code 0144 - Raising of Camels and Camelids", with a declared turnover of 219,134 lei/49,803 euros, a net profit of 14,048 lei/3,193 euros, and two employees.

Romania has a long-standing tradition in "Sheep and goat farming NACE Code 0145". There are 410 economic operators registered with this sector as their primary activity, with a combined turnover of 936.7 million lei/212.9 million euros (0.04% of the national total), a net profit of 36.1 million lei/8.2 million euros, and 737 jobs (0.02% of the national total).

The Top 5 counties by turnover include Brașov (142.9 million lei/32.5 million euros), Hunedoara (141.8 million lei/32.2 million euros), Timiș (119 million lei/27 million euros), Tulcea (103.3 million lei/23.5 million euros), and Mureș (93.7 million lei/21.3 million euros). Brașov, Hunedoara, and Timiș are counties with significant contributions to

the sector in terms of both the number of economic operators and turnover.

Romania is the leading exporter of live sheep in the European Union, with a total export value of 5,722,433 euros (912.9 tons, 6.27 euros/kg) (Profit.ro, 2024).

In the "Pig farming sector in Romania - NACE Code 0146", there are 527 economic operators (0.02% of the total economic operators in Romania) with a combined turnover of 3.1 billion lei/710.5 million euros (0.12% of the total national turnover), a net profit of 285.1 million lei/approximately 64.8 million euros, and 3,235 jobs (approximately 0.08% of the total workforce in Romania). Counties with significant impact on this sector include Vrancea (390.2 million lei/88.7 million euros), Vâlcea (360.2 million lei/81.9 million euros), Ilfov (236.7 million lei/53.8 million euros), Neamț (217.9 million lei/49.5 million euros), and Bihor (188.3 million lei/42.8 million euros). Although this sector represents a small share of the national economy, it covers part of the domestic pork consumption.

The "Poultry farming sector - NACE Code 0147" has shown significant growth in recent years. At the national level, this sector is the only one that has grown compared to the communist period (+60%) and covers domestic consumption, with surplus available for export. In 2023, Romania produced 723,000 tons of poultry meat (over 51% of the total meat production at the national level), with an average consumption level of 28.2 kilograms per capita per year (Cicovschi, 2024). The sector includes 626 economic operators (0.02% of the total), with a combined turnover of 6.6 billion lei/1.5 billion euros (0.26% of the country's total turnover), a net profit of 630.4 million lei/143.3 million euros, and 8,959 jobs. The Top 5 counties in terms of turnover and number of employees at the national level include Dolj (725.5 million lei/164.9 million euros), Vâlcea (506.2 million lei/115 million euros), Bihor (447.7 million lei/101.8 million euros), Brașov (402.4 million lei/91.5 million euros), and Prahova (369.4 million lei/84 million euros).

Although the sector's contribution to the national economy is small, its impact on national food security is significant, ensuring the population's needs for poultry meat and eggs.

Companies with their primary activity under "NACE Code 0149 - Raising of Other Animals" represent 478 economic operators (0.02% of the total economic operators in Romania), with a combined turnover of 118.5 million lei/26.9 million euros, a net profit of 19 million lei/4.3 million euros, and 462 employees (0.01% of the total workforce in Romania).

Economic operators from Brașov (23.8 million lei/5.4 million euros), Bucharest (23 million lei/5.2 million euros), Argeș (8.4 million lei/1.9 million euros), and Cluj (8 million lei/1.8 million euros) account for over 55% of the sector's turnover.

The main specific activities in this sector in Romania include beekeeping (for honey and other apicultural products), snail farming (for domestic consumption and export), silkworm farming (for natural silk production), fur animal farming (as long as it remains permitted nationally), and rabbit farming (for meat and fur). Starting in 2027, the farming of minks and chinchillas for fur will be banned in Romania (Petrache, 2024).

Associations and Producer Groups in Romania's Zootechnical Sector

The Ministry of Agriculture and Rural Development (MADR) provides a list of representative associations and federations in the animal husbandry sector (MADR, 2024). According to the information from the ministry, as of 2024, there are 38 representative associations and federations registered nationally in the field of zootechnics.

The distribution of associations based on the animal species they represent is shown in Figure 2.

Most associations are registered in the sheep and goat farming sector, followed by cattle farming and pig farming.

Across counties, the distribution includes only 35 counties (including Bucharest), each hosting at least one association in the field (Figure 3).

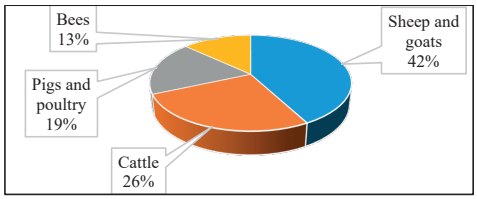


Figure 2. Associations by livestock activities
Source: Authors, by using MADR (2024)

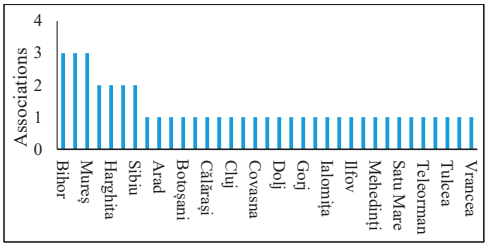


Figure 3. Associations by counties
Source: Authors, by using MADR (2024)

The counties of Bihor, Brașov, and Mureș each have three associations registered, while Bacău, Harghita, Neamț, and Sibiu each have two associations.

Some national federations have branches in multiple counties, which is why the total count of counties with at least one registered associative entity amounts to 45.

The counties of Brăila, Călărași, Galați, Sălaj, Satu Mare, and Vaslui have no registered organizations. Correlating information about zootechnical associations with data on economic agents under NACE codes reveals that the counties of Brașov, Mureș, and Bihor, frequently mentioned among the economic leaders in the zootechnical sector, also stand out by having a larger number of associations. Counties with significant activity in animal husbandry tend to be better represented through associative forms.

The presence of these organizations can serve as an indicator of the level of organization and access to resources, factors that directly contribute to the development of the zootechnical sector.

The calculation of the Gini-Struck Index (GSI) for the Romanian zootechnical sector yields a value of 0.081738553, which indicates an excessively diversified market. The calculation method and the interpretation of results were recommended by Săvoiu et al. (2010).

CONCLUSIONS

The research highlighted the role of the zootechnical sector in Romania's agricultural economy, both in terms of its contribution to GDP and its impact on rural development and food security. The relatively small number of associations and cooperatives compared to other European countries reflects the low level of organization and cooperation among domestic agricultural producers, which limits the sector's competitiveness. There is an uneven distribution of associations across counties, which may indicate a lack of representation in certain regions, potentially affecting farmers' ability to access development funds and markets. The low value of the Gini-Struck Index indicates excessive diversification and low concentration of associations, suggesting untapped potential for consolidation and collaboration.

There is significant growth potential for Romania's zootechnical sector through the optimal use of local resources, the promotion of animal welfare, and the adoption of modern technologies. However, government strategies and support measures are needed to encourage producer associations and to reduce gaps compared to practices in other European Union member states.

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TECHNOLOGIES OF THE AGRO FOOD PRODUCTS PROCESSING

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EVALUATION, MEASUREMENT AND STABILITY OF FRESH PRODUCT SHELF LIFE: IMPLICATIONS FOR REDUCING FOOD WASTE AND PROMOTING SUSTAINABLE FOOD PRODUCTION

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Abstract

This research analyses the assessment, quantification, and stability of fresh product shelf life, emphasizing their impact on food waste mitigation and sustainable food production. Given that food waste presents considerable environmental and economic concerns worldwide, it is essential to comprehend and enhance the shelf life of perishable goods. The research examines multiple evaluation techniques, such as shelf life testing, sensory assessments, and prediction modelling, to ascertain freshness and quality. Furthermore, it underscores the significance of sophisticated preservation methods, including active packaging and refrigeration technology, in prolonging shelf life and preserving product integrity. By enhancing shelf life, food makers, merchants, and consumers can more effectively manage stocks, decrease spoilage, and mitigate waste. This research seeks to enhance a sustainable food system by ensuring the delivery of fresh items to consumers while minimizing the ecological consequences of food waste.

Key words*: evaluation, food waste, shelf life, stability, sustainability.*

INTRODUCTION

Shelf life determination is a key aspect of food quality and safety assurance in putting safe and quality food on the market and also to assure a good food safety management. For an accurate estimation of foods shelf life, it is really important to first analyse the categories of food spoilage (physical, chemical and microbiological), the factors that influence the food spoilage and to identify the ways of controlling the food spoilage. Secondly, there is a need to identify the ways of measuring and predicting the shelf life and also to communicate it in order to raise awareness on food safety and also sustainability and food waste reduction.

Food waste represents a significant issue for society, with around 59 million tonnes being discarded annually across the European Union (https://food.ec.europa.eu/food-safety/food-waste_en).

In the context of food loss, the food industry generates significant amounts of byproducts each year during food processing. The most common are those derived from fruits and vegetables, which can be produced during pre-harvest and post-harvest stages, as well as throughout the preparation and processing phases.

These industrial by-products are very different from one another due to the difference in industrial processes (Nardella et al., 2022).

According to the European Commission, the main causes of food waste include a lack of awareness, poor shopping planning, confusion between "best before" and "use by" dates, and insufficient knowledge on how to make use of leftovers. Additionally, various studies have highlighted that a significant portion of household food waste results from preparing too much food and not consuming leftovers from previous meals (Lisciani et al., 2024).

Food waste is a pressing global issue, with implications that stretch across environmental, economic, and social dimensions. In industrialized nations, waste occurs at every phase of the food supply chain, starting from initial production losses on farms to consumer-end waste in retail environments. In the European Union alone, approximately yearly, the disposal of 59 million tons of food leads to significant resource loss, elevated greenhouse

gas emissions, and numerous missed opportunities for feeding those in need. To tackle this challenge, a dual approach is required, addressing both upstream (production and processing) and downstream (retail and consumer) inefficiencies (Redlingshofer et al., 2017).

At the retail level, food waste is often driven by misaligned inventory management, overstocking, and conservative shelf-life settings for perishable items. Traditional fixed shelf life (FSL) labelling can lead to premature disposal, especially for highly perishable goods. As a solution, dynamic shelf life (DSL) models offer a more flexible approach by adjusting expiration dates based on real-time product quality indicators, which can be combined with discounting strategies to incentivize consumer purchases of near-expiration items. Together, DSL and discounting have the potential to reduce waste significantly while maintaining food safety and maximizing retailer profits (Buisman et al., 2019).

Simultaneously, addressing waste at earlier stages in the supply chain - specifically during primary production and processing - is critical. Food loss during these stages, often overlooked, can stem from natural factors like weather, handling practices, and stringent quality standards. For instance, in sectors like fruits, vegetables, and cereals, between 3% and 12% of food can be lost before reaching consumers, highlighting the need for efficient loss quantification and targeted intervention at the production level.

This study combines insights from DSL and discounting practices in retail with quantification of production-stage losses in various food sectors, aiming to present a comprehensive strategy for food waste reduction. By examining these issues holistically, this research underscores the importance of integrated approaches that span the entire supply chain, supporting sustainable food systems and mitigating the negative impacts of waste on the economy and environment.

MATERIALS AND METHODS

This paper draws upon recent publications accessed through platforms such as Science Direct, Web of Science (via the Enformation platform), and Google Scholar. It also incorporates information from the websites of the Food and Agriculture Organization (FAO) and the European Food Safety Authority (EFSA), as well as national legislation from various countries related to the determination, assessment, and calculation of shelf life.

Additionally, two specific studies referring to (1) retail-level waste management through dynamic shelf life (DSL) and discounting strategies, and (2) quantification of food loss during primary production and processing stages were taken in the analysis. Combining these perspectives allowed us to give a holistic assessment of food waste reduction across the supply chain.

RESULTS AND DISCUSSIONS

1. Food spoilage: types and factors of influence

Food spoilage can have an important influence on food safety and quality and also the length of shelf life of foods (Basharat et al., 2022).

Physical spoilage refers to visible deterioration in food caused by factors such as physical damage, moisture loss or absorption, changes in texture, and other noticeable alterations in quality - often due to improper handling during storage, transport, or distribution. Fresh fruits and vegetables are particularly susceptible to such damage, which can occur as a result of poor respiration or chilling injuries during storage. Additionally, moisture transfer to or from food products can lead to spoilage and decay in many types of food (Roudaut et al., 2002). The low moisture content of powdered meals renders them very prone to absorbing moisture from their storage environment, resulting in an amorphous structure and causing the particles to adhere and form lumps. Crystal growth is another physical deterioration that can easily develop in food systems. The production of ice crystals from slow freezing or repeated freeze-thaw cycles in ice cream leads to a gritty texture in the final product (Ozmen & Langrish, 2002).

Chemical spoilage of food products can arise from the interaction of its chemical constituents or from their degradation influenced by factors such as temperature, water activity, pH, and storage conditions significantly influence food stability. The degradation caused by

interactions between sugars and proteins can be classified into enzymatic and non-enzymatic processes. In fresh fruits and vegetables, enzymatic reactions may result in tissue softening and the formation of brown pigments. The deterioration of lipids in food might result from oxidation, enzymatic degradation, or other hydrolytic reactions. In hydrolytic rancidity, the fatty acids separate from triglycerides due to the reaction of lipolytic enzymes in the presence of water. The resulting shorter-chain fatty acids tend to have a weaker flavor and can sometimes produce a rancid smell. These reactions typically take place at high temperatures, generally above 60°C, and can be prevented by reducing the exposure of lipid molecules to moisture (Kilcast & Subramaniam, 2000).

Microbial spoilage constitutes a prevalent and critical category of deterioration in all food kinds. It constitutes around 25% of global food loss and is associated with numerous foodborne illnesses (Bondi et al., 2014). A variety of microorganisms are recognized as agents of food deterioration and significant foodborne illnesses. Typical instances comprise *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Bacillus cereus*, *Campylobacter jejuni* and *Vibrio* spp. (Şuler et al., 2021) The incidence of food spoiling due to bacteria is significantly elevated, particularly in high-protein foods such as fish, dairy, and meat products (Petruzzi et al., 2017; Postolache et al., 2023). *Rhizopus* spp., *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. are the primary molds responsible for food deterioration, with certain *Aspergillus* species creating aflatoxins that contribute to foodborne sickness.

Food spoilage typically arises from both intrinsic properties of the food and diverse environmental influences. Raw foods possess inherent resistance against microbial infestation owing to the robust structure of their exteriors (such as fruit and vegetable peels, shells, and bran). Nonetheless, these goods sustain physical damage during many processing processes and are susceptible to microbial infestation and deterioration caused by enzymes (Petruzzi et al., 2017).

Foods high in sugar are typically susceptible to fermentative germs, while those rich in protein and fat are more likely to be affected by proteolytic and lipolytic microbes, respectively (Modi, 2009). Some spoiling reactions are catalysed by the presence of diverse metal ions and other trace elements. Conversely, the existence of several naturally occurring antimicrobial substances in foods might impede or diminish spoiling rates, hence prolonging product shelf life (Davidson & Critzer, 2012). Lysozyme found in egg albumin disrupts the cell walls of Gram-positive bacteria and inhibits protein breakdown. Additional antimicrobial food constituents that impede the proliferation of spoilage microorganisms include nisin, sorbates, citrate, butylated hydroxyanisole, benzoates, and sulphur dioxide (Jeantet et al., 2016).

Water activity (a_w) is defined as the ratio between the vapor pressure of a given substance and that of pure water under the same conditions. Most bacteria thrive in an optimal water activity range of 0.995 to 0.98. A minor decrease in water activity within the food system, either through moisture reduction or the incorporation of salt, sugar can significantly inhibit microbial development and substantially extend its shelf life. Bacteria often necessitate elevated water activity for growth and are not easily supported in desiccated food products (Modi, 2009).

pH quantifies the concentration of hydrogen ions in a substance, correlating to its acidity level. Each microbe possesses an optimal pH range for its development and metabolic functions: most of bacteria proliferate at neutral pH range (Jeantet et al., 2016), yeasts can proliferate within a pH range of 4.5-7.0, with certain species capable of thriving in a pH range of 2.0-2.5 (Modi, 2009).

Each microorganism grows within a specific temperature range, reaching its highest growth rate at its optimal temperature. Bacteria are classified as psychrophiles, mesophiles, or thermophiles, with optimal growth temperatures of approximately 12-15°C, 30-45°C, and 52-75°C, respectively. The lag phase of microbial development often diminishes as temperature rises, facilitating enhanced growth in future periods. A greater temperature concurrently influences various enzyme activity and protein synthesis, hence impacting the product's shelf life. Yeasts and molds,

conversely, may proliferate readily at or below ambient temperature and are frequently responsible for the deterioration of food stored in cold and chilly environments (Modi, 2009). Bacterial and fungal spores are regarded as the most heat-resistant species, with some enduring temperatures exceeding 100°C (Jeantet et al., 2016).

2. Evaluation, detection and identification of food spoilage

Food spoilage refers to the decline in nutritional value, sensory qualities, and overall safety of food, making it unsuitable for consumption. To prevent spoilage, it is essential to understand the microorganisms responsible and to implement various systems, methods, and technologies. At the industrial level, spoilage is managed or detected through the application of the Hazard Analysis Critical Control Point (HACCP) system, along with efficient production practices and thorough risk assessments (Panisello & Quantick, 2001). Heat, packaging, processing, and storage for food preservation diminish the likelihood of spoiling causes in the environment.

The detection and identification of spoiling are critical criteria in contemporary food industries due to health hazards linked to the consumption of infected foods are potentially fatal.

Biosensors are analytical devices that integrate a biological recognition element with a physicochemical transducer for the detection of specific chemical substances. Recent methodologies have employed enzymatic reactor systems coupled with amperometric electrodes to quantify diamine concentrations, thus enabling the evaluation of meat quality, particularly in beef, fish, and poultry. Postmortem enzymatic activity further contributes to the degradation of muscle tissue. Moreover, advanced biosensing platforms based on nucleic acid probes (DNA or RNA), mass spectrometry, bioluminescence, and related technologies are currently under development for the rapid and accurate detection of bacterial contamination in food products (Freitas et al., 2011); nevertheless, progress in mold detection remains limited.

Microbial metabolites serve as possible indicators for detecting food decomposition in meat products. After bacteria have utilized surface-level glucose, they will subsequently metabolize various substrates, including free amino acids. To do this, numerous bacteria excrete various proteolytic enzymes, with Gram-negative bacteria mostly releasing amino peptidases at refrigeration temperatures on meat (Basharat et al., 2022). Numerous researchers have suggested that the involvement of enzymes may be swiftly utilized using enzyme assays to assess beef stability about bacterial counts. The synthesis of amines, ammonia, indoles, and other pH-lowering chemicals further exacerbates deterioration due to amino acid consumption.

A developed tool is the nucleic acid-based detection of spoilage microorganisms, referred to as polymerase chain reaction (PCR). This technique identifies many gene sequences that specifically assist in detecting rotting microorganisms. The application of PCR-based typing techniques, including microsatellite PCR fingerprinting and random amplified polymorphic DNA (RAPD) analysis (Basharat et al., 2022), enables the differentiation of individual spoilage strains, regardless of species, and facilitates the identification of their sources of origin (Basharat et al., 2022). A listing for this kit for the identification of fungi in food by DNA fingerprinting is found in Fung (2002); however, no recent activity regarding its recognition is reported. A DNA-based approach for detecting molds in food may become commercially viable with additional development.

Spoilage can be identified through alterations in organoleptic qualities, including changes in odor, the presence of visible microflora colonies, surface stickiness of meat, and variations in flavor. The alterations in organoleptic odor are currently assessed by electronic noses and have shown highly effective in the evaluation of meat and fish (Gardner & Bartlett, 2000). The identification of volatiles as an indicator of mold growth was utilized in the development of electronic noses. Söderström et al. (2003) conducted an experiment to measure mold development in malt extract solution via an electronic tongue, akin to an electronic nose, which has several metal sensors that detect non-volatile substances in liquid.

3. Modern Measurement Techniques

Accelerated Shelf Life Testing (ASLT)

ASLT involves subjecting products to intensified stress conditions - such as elevated temperature and humidity - to speed up the degradation process. The data obtained is then analyzed using the Arrhenius equation to estimate the product's shelf life under typical storage conditions (Calligaris et al., 2019). For instance, a product stored at 40°C for 3 months may simulate 12 months of shelf life at 25°C.

Predictive Modelling

Predictive models use mathematical equations to forecast shelf life based on degradation kinetics. Common models include zero-order, first-order, and Weibull kinetics. Software tools, such as ComBase and Pathogen Modeling Program (PMP), integrate microbial growth and chemical reaction data to predict shelf life (Cakmak, 2019).

Non-Destructive Techniques

Non-destructive methods allow real-time monitoring without altering the product: Near-Infrared Spectroscopy (NIRS): Measures chemical composition and moisture content; Electronic Noses (E-Noses): Detect volatile compounds indicative of spoilage; Magnetic Resonance Imaging (MRI): Visualizes internal structural changes.

Biosensors and Smart Packaging

Biosensors detect specific biomarkers of spoilage, such as pH changes or microbial metabolites. Smart packaging incorporates indicators (e.g., time-temperature indicators, freshness sensors) that provide real-time information on product quality (Yam et al., 2005).

4. Predicting shelf life

Predicting shelf life accurately is crucial for minimizing food waste and ensuring food safety and quality. It involves using various scientific methods and models to estimate the time a food product remains acceptable for consumption.

This prediction is based on different methods based on different factors that influence the shelf life (based on water activity, pH, nutrient content, enzyme activity, food matrix and food structure and antimicrobial compounds, temperature, humidity, light exposure). These are real-time storage studies and accelerated shelf life tests which are time and resource consuming (Chanpet et al., 2020).

The traditional methods depend on sensory assessment, expertise, and historical information. Although beneficial, they are inherently subjective and lack precision. Accelerated shelf life studies subject the food to more severe circumstances (e.g., elevated temperatures) than typical storage practices. The observed degradation rates are extrapolated to forecast shelf life under standard storage circumstances. This is predicated on the assumption that the degradation mechanisms adhere to Arrhenius kinetics (www.hemdahl.com).

Predictive microbiology models employ mathematical frameworks to characterize the proliferation of microbes under certain parameters (temperature, pH, aw, etc.). These models can use intrinsic and extrinsic elements to mimic microbial development and forecast the duration required to attain a given spoiling level (e.g., a designated microbiological count or sensory threshold) (Stavropoulou et al., 2019).

Kinetic modelling of chemical reactions akin to predictive microbiology, simulates the pace of chemical reactions that lead to food degradation (e.g., oxidation, enzymatic processes). These models are employed to forecast alterations in food quality characteristics (e.g., color, flavor, texture) across time (van Boekel, 2009).

Machine learning and artificial intelligence: These sophisticated methodologies may evaluate extensive datasets of intrinsic and extrinsic variables alongside observed shelf life data to construct predictive models. They can discern intricate correlations between variables and shelf life, which are challenging to encapsulate using conventional models. Algorithms such as support vector machines (SVMs), neural networks, and random forests are frequently employed (Shi et al., 2023).

5. The design of shelf life experiments

The ideal approach to sensory testing involves assessing all samples from each storage condition and time point simultaneously within a balanced design. This can be achieved in three different ways. First, samples can be collected from successive production batches

and stored for a specific period. Once they reach the end of their shelf life, all samples can be evaluated together using an appropriate testing setup (Figure 1). However, this method is susceptible to variations in production quality and is only suitable when production consistency is assured (Kilcast et al., 2000).

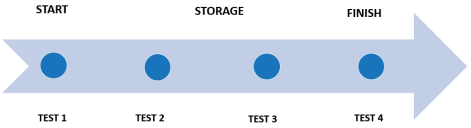


Figure 1. A shelf life testing strategy based on a partially staggered design

Another version is conducted wherein a single large batch is maintained under circumstances that ensure negligible quality alterations, such as frozen storage. Samples are extracted at specified intervals and preserved under optimal conditions (Figure 2).

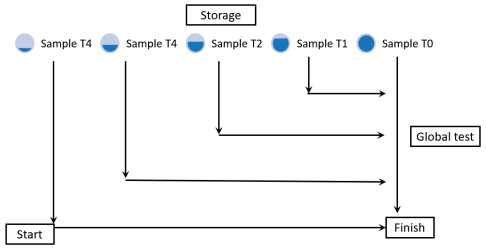


Figure 2. Simple design for shelf life testing

The third form (Figure 3): a significant batch is preserved, and samples are retrieved at specified intervals and kept under stable conditions (e.g., frozen) until the necessary storage period is fulfilled. The primary challenge in the latter two designs lies in determining suitable stable storage conditions, given that most food products cannot be stored for extended periods without significant changes in quality attributes. While all three methodologies offer the substantial benefit of delivering an internally consistent depiction of changes in sensory attributes, they are impeded by several limitations. Initially, no data regarding stability and shelf life is generated until the completion of storage studies, a situation unlikely to be tolerated in most industrial environments. Secondly, initiating the trials requires prior knowledge of the expected shelf life. Thirdly, a worldwide assessment may pose considerable logistical difficulties for instrumental laboratory measurements (Kilcast et al., 2000).

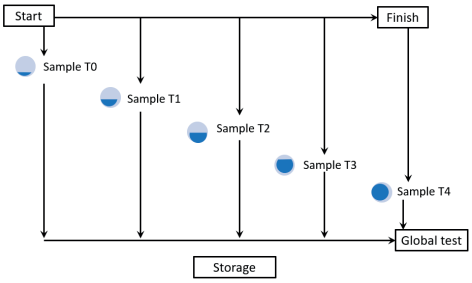


Figure 3. Another design for shelf life testing

6. Retail-Level Waste Reduction Through Dynamic Shelf Life (DSL) and Discounting

The implementation of dynamic shelf life (DSL) systems at the retail level has demonstrated significant potential for reducing food waste, particularly for highly perishable items. DSL operates by adjusting expiration dates in real time, based on factors such as temperature and product quality, allowing retailers to keep products on shelves longer when conditions permit. This flexibility contrasts with fixed shelf life (FSL) systems, which often lead to early disposal due to conservative expiration settings (Redlingshofer et al., 2017).

Key results from the simulation model reveal that DSL:

- Reduces Waste by Extending Usable Shelf Life: Compared to FSL, DSL allowed products to stay on shelves longer, resulting in a 10-20% decrease in waste. (Stenmarck et al., 2016). By continuously assessing product quality, DSL ensures that products are disposed of only when they no longer meet safety standards.
- Increases Retail Profitability: With DSL, retailers experienced a profit increase due to fewer losses and improved stock turnover. Profit gains were especially notable when DSL was paired with discounting, which encouraged consumers to purchase near-expiration items.
- Reduces Stock Shortages and Improves Inventory Planning: DSL's adaptability enabled

more accurate replenishment, helping retailers avoid overstocking and unexpected stock-outs. Discounting further enhanced these outcomes by aligning with consumer purchasing behavior. Products near expiration were marked down, making them more attractive to price-sensitive customers. The combined use of DSL and discounting resulted in optimal reductions in waste while preserving or even boosting retailer profits (Buisman et al., 2019). Overall, these findings suggest that DSL, particularly when coupled with targeted discounting, provides a robust framework for minimizing waste and maximizing sales of perishable goods. Retailers can adjust these strategies based on seasonal demand and product type, further supporting inventory flexibility.

7. Quantification of Food Losses in Primary Production and Processing

The quantification of food losses at the stages of primary production and processing reveals considerable waste across diverse segments of the food system, underscoring critical intervention points for improving efficiency and sustainability.

- Fruits and Vegetables: Losses in this sector were the highest, averaging up to 12%. Contributing factors include handling damage, spoilage from delays, and high grading standards that lead to the discard of visually imperfect items.
- Cereals and Pulses: Loss rates for cereals and pulses were between 3% and 7%. Losses were often due to environmental factors such as weather variability, as well as handling and storage practices.

The findings indicate that food losses vary widely depending on the type of product and processing requirements, suggesting that sector-specific waste reduction measures are essential. Improving handling practices, upgrading storage facilities, and adjusting visual quality standards could significantly reduce waste, particularly for fresh produce.

8. Recycling and Reuse as a Supplementary Waste Reduction Strategy

The study also highlights the importance of recycling and reuse to minimize waste across the supply chain. When products are unsuitable for direct consumer sales but still safe for consumption, they are often diverted to animal feed or food donations. Expanding food donation programs, particularly for fruits and vegetables, could further reduce food loss while supporting communities in need. However, the effectiveness of these efforts depends on well-coordinated logistics and partnerships between producers, retailers, and charitable organizations.

The main goal in preventing food waste should be to take action directly at the source by reducing the production of surplus food at every stage of the food supply chain - namely production, processing, distribution, and consumption. When this is not possible, the next best preventive measure, aligned with the waste hierarchy and aimed at maximizing the value of edible food, is to redirect surplus food for human consumption, provided it is safe to do so. As part of the Circular Economy Action Plan, the European Commission has introduced EU guidelines on food donation to support the recovery and redistribution of safe, edible food to people in need. These guidelines, developed in collaboration with the EU Platform on Food Losses and Food Waste, aim to:

- Support both food donors and recipients in complying with EU regulations (including those on food safety, hygiene, traceability, liability, and VAT);
- Encourage a consistent interpretation of EU rules across Member States regarding the redistribution of surplus food. (https://commission.europa.eu/index_en) (Figures 4 and 5).

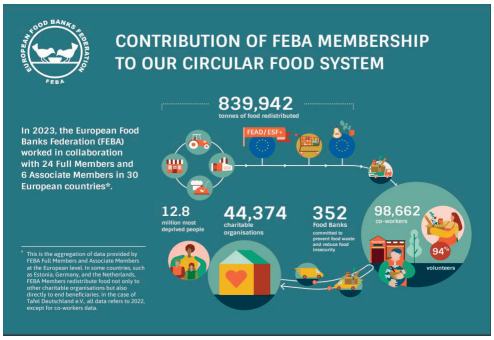


Figure 4. Contribution of FEBA as supplementary waste reduction strategy.
Source: https://www.eurofoodbank.org

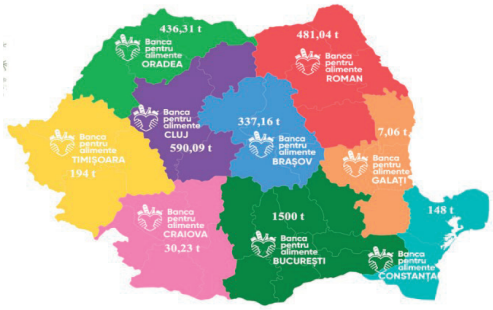


Figure 5. Network of Food Banks in Romania and the quantities (tons) of food collected in 2021. Source: https://rocesp.ro/

9. Integrated Impact Across Supply Chain Stages

By combining DSL and discounting strategies at retail with quantification of production-level losses, this study provides an integrated approach to reducing waste across the food supply chain. While DSL and discounting address waste at the consumer end, quantifying early-stage losses in production and processing enables targeted interventions for specific sectors. For example, introducing better storage and handling methods at the production level can help ensure that produce reaches retailers in optimal condition, reducing waste before it even reaches the shelf.

This integrated strategy reveals that effective food waste management must span the entire supply chain, from production to consumer. Retail interventions like DSL and discounting are valuable for perishable items close to the point of sale, while improvements in primary production can help prevent waste upstream. Together, these findings suggest that combining downstream and upstream approaches can maximize food waste reduction across diverse food categories (Buisman et al., 2019).

CONCLUSIONS

Extending the shelf life of fresh products is essential for reducing food waste and advancing food sustainability. This study demonstrates that dynamic shelf life (DSL) systems, especially when combined with discounting, can significantly decrease waste in retail by adjusting expiration dates based on real-time quality assessments. DSL allows retailers to maintain products on shelves as long as they are safe, preventing premature disposal and increasing profitability. For highly perishable items like fruits, vegetables, DSL enhances inventory flexibility, reducing the risks of spoilage and overstocking.

Beyond retail, addressing waste during production and processing stages is critical. Quantifying these losses identifies areas where improved handling, storage, and grading standards can reduce waste at the source, ensuring that more fresh products reach consumers. Integrating DSL with early-stage loss prevention supports a more sustainable food system, conserving resources, reducing emissions, and enhancing food security. Moving forward, innovations in shelf life extension and consumer acceptance of "imperfect" produce will be vital for a circular, sustainable food economy focused on minimizing waste from farm to table.

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ASSESSMENT OF NUTRITIONAL AND FUNCTIONAL PROPERTIES OF YOGURT ENRICHED WITH ARONIA POMACE POWDER

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Abstract

Aronia pomace powder, obtained as a by-product of juice production through pulp exhausted, is rich in bioactive compounds like anthocyanins, flavonoids, and polyphenols, known for their antioxidant and health-promoting properties. Incorporating aronia pomace powder (APP) into yogurt at 4% and 6% levels offers a novel approach to enhancing its nutritional, sensory, and functional attributes. This study evaluated the effects of Aronia powder on yogurt's physicochemical properties, phytochemical profile, texture, color and sensory acceptability. APP-enriched yogurts exhibited increased total phenolic content (2.50-2.94 mg GAE/g) and antioxidant activity (17.47-19.27 µmol Trolox/g), alongside a visually appealing reddish-purple color due to anthocyanins. The sensory evaluation highlighted 6% APP as the optimal concentration, balancing taste and nutritional benefits. Furthermore, APP demonstrates significant potential for the development of functional dairy products, aligning with consumer preferences for health-oriented and sustainable options.

Key words*: antioxidants, Aronia by-products, functional dairy products, polyphenols, value-added ingredient.*

INTRODUCTION

The demand for functional foods with health benefits has risen, with fruit-enriched dairy products being among the most popular due to their bioactive content and consumer preference (Rincón-León, 2003). Berries such as blackberries, raspberries, black currants, blueberries, and elderberries are rich in phenolic compounds and fiber, making them ideal for incorporation into dairy products like kefir, yogurt, and fermented milk (Ozdemir & Ozkan, 2020).

The use of fruit pomace enhances the circular economy, while natural antioxidants align with the clean label trend (Raikos et al., 2018). The nutritional value and bioactivity of yogurt are further improved by adding fruit and fruit juices (Dimitrellou et al., 2020; Plessas et al., 2024). Yogurt, known for its nutritional and sensory qualities, has been widely studied for functional enhancements, benefiting both the dairy industry and consumers by extending shelf life and offering higher energy and superior sensory profiles (Kowaleski & Battestin, 2020). The fortification of yogurt with bioactive a superfood with strong antioxidant qualities, Aronia (*Aronia melanocarpa*) berries, also known as black chokeberries, contain many bioactive compounds, including anthocyanins, carotenoids, fatty acids, flavonoids, phenolic compounds, and vitamins. The fruit has recently attracted attention because of its powerful antioxidant activities (Peng et al., 2022). Aronia pomace powder (APP), derived from aronia juice processing, contributes to functional food development and circular economy (Lazăr & Catană, 2020).

We anticipate that adding powdered aronia pomace to yogurt will enhance dietary fiber and polyphenol intake, lower fat content without altering texture, and ensure product atractivity. This study evaluates the impact of 4% and 6% APP supplementation on yogurt's

physicochemical, textural, antioxidant, and sensory properties.

MATERIALS AND METHODS

According to the research methodology, the first stage included qualitative evaluations of the powder from a phytochemical point of view and of the raw material - in this case, milk. The final products were analyzed from a chemical, phytochemical, physical and sensory point of view and the technological processes involved in the production of yogurt were described.
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Obtaining powder from Aronia pomace powder

To obtain *Aronia melanocarpa* chokeberry powder, the fruits were washed, squeezed and the resulting chokeberry dried at +42°C to protect the bioactive compounds (anthocyanins, flavonoids, polyphenols). Once dried, the chokeberry was ground and turned into a fine powder using a high-speed grinder.
The **total anthocyanin content** was determined using the modified pH differential method described by Lipsa et al. (2024). Samples were diluted at a 1:10 ratio, and a reaction mixture consisting of 200 µL vegetable extract and 800 µL buffer solution (pH 1.0/4.5) was prepared. Absorbance was measured at 520 nm and 700 nm using a UV-Vis spectrophotometer (Analytik Jena-Specord 210 Plus, Jena, Germany). The results were expressed as milligrams of cyanidin-3-glucoside (C3G) per gram of dry matter (dm).
The **total flavonoid content** was determined using the aluminum chloride spectrophotometric method described by Lipsa et al. (2024). The reaction mixture consisted of 0.25 mL extract, 2 mL distilled water, and 0.075 mL 5% sodium nitrite, followed by the addition of 0.15 mL aluminium chloride after 5 minutes and 0.5 mL 1 M sodium hydroxide after 6 minutes. Absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Analytik Jena Specord 210 Plus, Germany). The total flavonoid content was expressed as mg catechin equivalents per gram (mg CE/g dm), based on a calibration curve (0.1-0.5 mg/mL, $R^2 = 0.997$).
The Folin-Ciocalteu method (Gavril et al., 2024) combined with spectrophotometric analysis was used to determine **the total polyphenol content** of the extract. The reaction mixture, consisting of 200 µL extract, 1 mL Folin-Ciocalteu reagent, 15.8 mL distilled water, and 3 mL 20% Na_2CO_3, was incubated for 60 minutes in the dark at room temperature. Absorbance was measured at 765 nm using an Analytik Jena Specord 210 Plus UV-VIS spectrophotometer (Germany). Polyphenols content was expressed as mg gallic acid equivalents per gram (mg GAE/g dm), based on a calibration curve (0.1-0.5 mg/mL, $R^2 = 0.984$).
The antioxidant activity of the extract was evaluated using the DPPH radical scavenging method, following the protocol of Lipsa et al. (2024). A reaction mixture containing 100 µL extract and 3.9 mL of 0.1 M DPPH solution was incubated in darkness at +25°C for 30 minutes. Absorbance was measured at 515 nm using a UV-Vis spectrophotometer (Analytik Jena-Specord 210 Plus, Germany). A control sample, consisting of 3.9 mL of 0.1 M DPPH solution and 100 µL methanol, was prepared under identical conditions. Antioxidant activity was expressed as µmol Trolox/g dm, based on a calibration curve

Preparation and caracterization of supplemented yogurt

The functionality of enriched yoghurt was assessed by incorporating Aronia pomace powder (APP) at two specific concentrations: 4% and 6%. A control group consisting of yoghurt without APP addition was also prepared for comparison. The selection of the 4% and 6% APP concentrations was guided by prior sensory evaluations conducted by a panel of experts, which identified these levels as optimal in terms of visual appeal, texture, flavour, and colour. The technological process for producing the enriched yoghurt is illustrated in Figure 1. The yoghurt production process involved the reception and filtration of raw

milk, followed by pasteurization at 90°C for 30 minutes to ensure microbiological safety. After cooling to 45°C, starter cultures (*Streptococcus thermophilus* YF-L81 and *Lactobacillus delbrueckii* subsp. *bulgaricus*) were inoculated, initiating fermentation. *Aronia melanocarpa* powder was added to develop YAPP4 (4% APP), YAPP6 (6% APP), and YM (without APP). The mixtures were thermosealed at 227°C and fermented at 43°C, facilitating lactose conversion. Post-fermentation, the yoghurt was cooled (20°C → 3°C) and stored at 3°C, preserving stability and sensory attributes. The enriched yoghurt was assessed for physicochemical parameters (fat, protein, ash, moisture, total dry matter, carbohydrates), using AOAC 2000 methods.

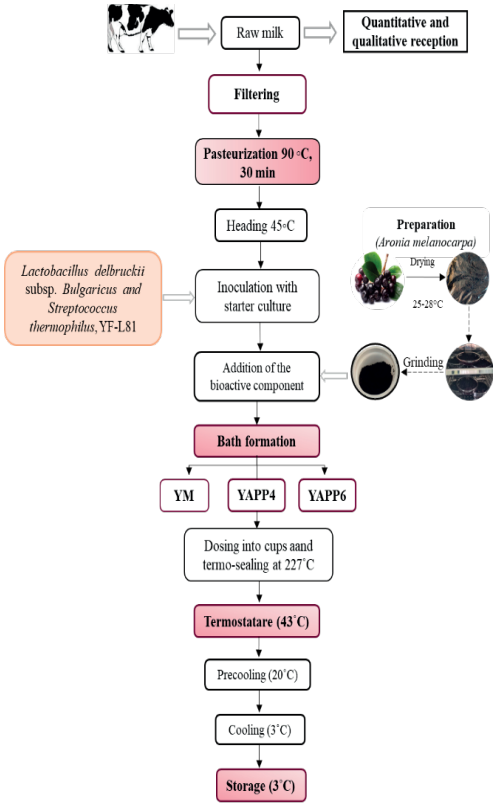


Figure 1. Functional yogurt prototype processing flow diagram (own source)

Phytochemical characterization of supplemented yogurts

The anthocyanin, flavonoid and phenolic content, as well as the antioxidant activity of APP-enhanced yogurt were evaluated using the procedures described above.

Color assessment of supplemented yogurts

According to Dag et al. (2017), the CIELAB system (L*, a*, b*) with a MINOLTA Chroma Meter CR-410 (Konica Minolta, Osaka, Japan) was used to analyze the color characteristics of Aronia pomace and APP enhanced-yogurts. To provide insight into the visual consequences of APP enrichment, L* denotes brightness, a* measures red-green tones, and b* depicts yellow-blue tones.

Textural parameters of supplemented yogurts

A Mark 10 ESM 300 texturometer (New York, NY, USA) fitted with a 5-50E series digital dynamometer (resolution: 0.05 N) was used to analyze the texture of enhanced yogurt samples.

Sensory evaluation of supplemented yogurts

The sensory evaluation process was conducted in strict accordance with the guidelines set in ISO 13299.

RESULTS AND DISCUSSIONS

With a high concentration of phenolic compounds and strong antioxidant activity, aronia powder has a complex phytochemical profile that makes it a useful component of nutraceuticals and functional foods. As presented in Table 1, its antioxidant and anti-inflammatory properties are attributed to the presence of flavonoids (9.85 ± 0.36 mg EC/g) and total anthocyanins (1.08 ± 0.02 µg/g), while total polyphenols (20.89 ± 0.12 mg) improve cellular protection and oxidative stability. Its high antioxidant activity (87.5 Trolox equivalents) further supports its nutritional and physiological significance by confirming its potential to scavenge free radicals. Anthocyanin content affects the powder's red color, as shown by a positive a value* in colorimetric analysis, whereas a yellow undertone* is suggested by the b parameter. The red-yellow color profile that is typical of anthocyanins is confirmed by the plotting of the combined +a and +b values** in the first quadrant. These results emphasize the dual benefits of Aronia powder as a natural colorant and a potent antioxidant source, which makes it a great option for food compositions that promote health.

Tabel 1. Phytochemical content and color parameters of the APP

Parameters		APP
Total Anthocyanins, µg/g dm		1.08±0.02
Total Flavonoids, mg CE/g dm		9.85±0.36
Total Polyphenols, mg GAE/g dm		20.89±0.12
Antioxidant Activity (DPPH)	µmol Trolox/g d.m	23.63±0.11
	Inhibition, %	87.54±0.36
L*		24.095±0.20
a*		12.635±0.05
b*		3.43±0.07

Yogurt's physical and nutritional qualities were improved by the substantial physicochemical changes brought about by the inclusion of aronia pomace, as detailed in Table 2. The increase in pH suggests low acidity, probably caused by interactions between phenolic compounds and lactic acid bacteria during fermentation The nutritional enrichment was confirmed by the nearly twofold increase in protein content (5.64%) and the rise in fat content from 4.26% (YM) to 5.48% (YAPP 6%). The fiber content increased from 0% (YM) to 3.83% (YAPP 6%), which may have functional advantages. Consistent mineral retention was indicated by the steady levels of carbohydrates (5.1%) and ash content (0.93% to 0.98%). Texture and stiffness were enhanced by the moisture reduction (84.99% to 78.89%), which also increased total dry matter (13.01% to 21.06%). Furthermore, the product's increased nutritional density was reflected in the energy value, which increased from 69.62 kcal/100 g (YM) to 100.14 kcal/100 g (YAPP 6%). According to these results, yogurt with aronia is a functional ingredient that is nutritionally superior due to its higher protein, fiber, and bioactive content.

In Table 3, it can be seen that the bioactive chemical profile of yogurt was significantly improved by enriching it with 4% and 6% aronia pomace, as shown by the notable rise in anthocyanin content from 0.76 to 1.16 µg/g and flavonoid concentration from 0.49 to 2.08 mg CE/g dm.

Table 2. Physical-chemical composition of plain enhanced yogurt

Physical-chemical characteristics	YM	YAPP4	YAPP6
pH	4.59±0.06	5.10±0.02	5.52 ±0.01
Fat, %	4.26±0.03	4.34±0.09	5.48±0.016
Protein, %	2.59±0.02	4.66±0.05	5.64±0.06
Carbohydrates, %	5.23±0.03	5.08±0.04	5.13±0.05
Fibre, %	-	1.95±0.07	3.83±0.12
Ash, %	0.93±0.01	0.94±0.08	0.98±0.12
Moisture, %	86.99±0.21	82.99±0.23	78.94±0.34
Total dry matter, %	13.01±0.03	16.97±0.04	21.06±0.07
Energetic value, kcal/100 g	69.62±0.02	81.92±0.03	100.14±0.02

Table 3. Phytochemical profile of plain and enhanced yogurts

Yogurt samples	Total anthocyanins, µg/g dm	Total flavonoids, mg CE/g dm	Total polyphenols, mg GAE/g dm	Antioxidant activity (DPPH)	
				µmol Trolox/g dm	Inhibition, %
YM	-	0.49±0.02	0.81±0.04	6.53±0.12	7.38±0.08
YAPP4	0.76 ±0.05	1.64 ±0.02	2.50 ±0.02	17.47±0.19	29.00±0.32
YAPP6	1.16 ±0.01	2.08 ±0.03	2.94 ±0.03	19.27±0.20	32.09±0.33

This implies that these physiologically active substances, which are well-known for their antioxidant and health-promoting qualities, are abundant in the added aronia powder. It's interesting to note that the highest total polyphenol content was at 6% addition (2.94 mg GAE/g dm), followed by 4% addition (2.50 mg GAE/g dm) in comparison to plain yogurt (0.81 mg GAE/g dm). This variation suggests possible interactions between the yogurt matrix and polyphenols. Most significantly, the addition of aronia powder resulted in a significant rise in antioxidant activity, with values increasing from 7.38% to 32.09%,

indicating the enriched yogurt's functional potential. Although the antioxidant effects are maximized at 6% addition, more research is needed to increase product stability and sensory acceptability in order to guarantee its feasibility for commercial uses. Our results were in line with flavored yogurts that also contained edible plant components high in anthocyanins, such as roselle (Leyva et al., 2013) and grape pomace (Demirkol et al., 2018) in their composition. These useful food pigments were added to yogurt for research since it has a low pH and promotes anthocyanin content.

Color is a key physical determinant of a dairy's quality. The results of measurements of the samples' hue angle, chroma, and color parameters L*, a*, and b* are shown in Table 4.

Table 4. Color parameters of plain and enhanced yogurts

Parameters	YM	YAPP4	YAPP6
L*	94.75±0.02	59.79±0.05	40.74±0.09
a*	-9.35±0.09	14.47±0.22	20.97±0.27
b*	16.58±0.14	21.25±0.29	31.14±0.32

The addition of aronia powder to the yogurt matrix resulted in notable chromatic parameter changes in the CIELab* system, including a notable drop in lightness (L*) from 94.75 (YM) to 59.79 (YAPP4) and 40.74 (YAPP6), indicating an intensification of the product's dark color. Anthocyanin pigments are responsible for the accentuation of the reddish hues, as seen by the concurrent rise in the a* component value from -9.35 (YM) to 14.47 (YAPP4), and 20.97 (YAPP6). Meanwhile, a drop in the yellow tones is indicated by the b* values going from 16.58 (YM) to 21.25 (YAPP4) and 31.14 (YAPP6), which further supports the red-bluish spectrum's dominance. The final product's visual attractiveness and sensory perception are affected by these chromatic fluctuations, which are directly connected to the amount of aronia pomace utilized. Our results were consistent with those of the study by Postolache et al. (2023).

The results of the texture profile analysis are shown in Table 5. The most important factor in establishing the texture of yogurt is its hardness. It measures the yogurt's hardness and is thought of as the force needed to cause a specific deformation. As the concentration of the additional powder increased, so did the hardness of the yogurt augmented with the addition. Because of its higher water-holding capacity, the dietary fiber from aronia powder may absorb more moisture, increasing the samples' hardness. These alterations could be linked to the high water absorption capacity of the powder fibers and interactions between lactic proteins and aronia bioactive chemicals. In contrast to the control sample, the yogurts enriched with Aronia pomace showed increased hardness, gumminess, cohesiveness, and adhesiveness and decreased springiness.

Table 5. Textural parameters of plain and enhanced yogurts

Textural Parameters	Sample	Value
Cohesiveness	YM	0.29±0.00
	YAPP4	0.35±0.01
	YAPP6	0.36±0.01
Springiness	YM	0.50±0.02
	YAPP4	-0.02±0.00
	YAPP6	-0.28±0.01
Hardness, N	YM	5.65±0.00
	YAPP4	15.68±0.02
	YAPP6	19.77±0.02
Gumminess, N	YM	1.63±0.01
	YAPP4	5.22±0.03
	YAPP6	6.36±0.04
Adhesiveness, mJ	YM	−2.51±0.01
	YAPP4	-29.56±0.01
	YAPP6	-47.70±0.00

The effect of aronia on sensory qualities as appearance, color, scent, taste, odor, and general acceptability is demonstrated by the hedonic evaluation of yogurts enhanced with aronia powder (YAPP4 and YAPP6) in comparison to the control (YM). From Figure 2, we can deduce that yogurts YAPP4 and YAPP6 both scored lower on apperence and color, suggesting that aronia's high pigmentation affects how the eye perceives it. The presence of phenolic chemicals, which can impart bitter or astringent overtones, is probably the reason why YAPP6 had a far lower taste score than YM. Furthermore, YAPP6 showed decreased consistency, indicating that texture is influenced by polyphenol-protein interactions. However, both YAPP4 and YAPP6 gave scent and odor high ratings, suggesting that aronia supplementation had no detrimental effects on these qualities. Moreover, YAPP4 was favored above YAPP6, indicating that altering the amount of Aronia powder could improve sensory balance.

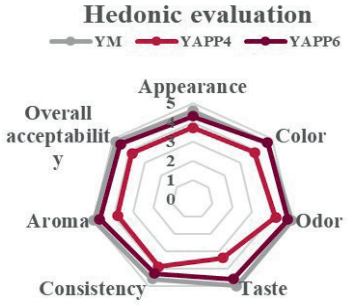


Figure 2. Comparative chart of the sensory attributes

CONCLUSIONS

APP has shown itself a bioactive ingredient when added to yogurt formulations, providing both functional and sensory advantages. Yogurt's total phenolic content and antioxidant capacity are greatly increased by APP addition compared to the control sample. Although increased APP concentrations may affect flavor and texture, sensory data indicates that consumers generally still find by-products enhanced yogurts to be satisfactory.

APP also offers the dairy industry a good source of anthocyanins, which makes it possible to create novel and health-promoting functional yogurts. To find the right incorporation levels and strike the perfect balance between nutritional advantages and sensory acceptability, more research is necessary.

Furthermore, the use of APP supports the development of eco-friendly technologies in the food business and is consistent with sustainable food production methods by encouraging the value-adding of agricultural byproducts.

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INNOVATIVE PACKAGING AND LABELING SOLUTIONS FOR PRESERVATIVE-FREE READY-TO-EAT MEALS: ENHANCING SHELF LIFE AND SUSTAINABILITY - STUDY CASE ROMANIA

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Abstract

The increasing consumer demand for healthier ready-to-eat (RTE) meals with fewer or no preservatives has created a pressing need for innovative packaging and labeling solutions that maintain product quality and extend shelf life. This study investigates advanced packaging technologies, including active packaging, modified atmosphere packaging (MAP), and nanotechnology-based materials, to minimize microbial growth and preserve sensory attributes. Additionally, the integration of intelligent labeling systems, such as time-temperature indicators (TTIs) and freshness sensors, is explored to enhance consumer confidence and reduce food waste. The review also evaluates the environmental implications of these solutions, prioritizing sustainable materials and design. By synergizing cutting-edge packaging methods with transparency in labeling, this study aims to develop a comprehensive framework for enhancing the safety, quality, and sustainability of RTE meals. Particularly, some good practices related with enhancing shelf life and sustainability for RTE meals in Romania are addressed in this paper.

Key words*: ready-to-eat meals, active packaging, intelligent labeling, shelf-life extension, sustainable materials.*

INTRODUCTION

Innovative packaging solutions are emerging to extend the shelf life of ready-to-eat (RTE) meals and improve sustainability. Active packaging technologies, such as antimicrobial films using natural compounds like turmeric and red ginger, can significantly prolong the shelf life of RTE meat products (Dharma, 2023). Intelligent packaging systems provide information on product quality and safety (Janjarasskul and Suppakul, 2017). Vacuum-sealed packaging in retort pouches enhances food safety against microbial contamination (Dharma, 2023). Sustainable or "green" packaging using edible or biodegradable materials, plant extracts, and nanomaterials can reduce environmental impacts (Han et al., 2018). These technologies can work synergistically to create multipurpose food-packaging systems (Han et al., 2018). For cook-chill foods, various compatible packaging systems and active packaging strategies are designed to increase shelf life (Clodoveo et al., 2021). These innovations address consumer demands for safe, high-quality, and environmentally friendly food products.

Recent research highlights innovative packaging technologies to extend shelf life and improve the safety of ready-to-eat (RTE) and home meal replacement (HMR) products. Active packaging strategiescan prolong product shelf life (Bumbudsanpharoke et al., 2022; Clodoveo et al., 2021). Specific examples include using turmeric and red ginger as antimicrobial film coatings for RTE meat products, potentially extending shelf life up to 30 days at room temperature (Dharma, 2023). Intelligent packaging technologies, like colorimetric indicators and RFID labels, enable real-time monitoring of food conditions (Bumbudsanpharok et al., 2022). Future packaging innovations aim to address food waste reduction, food safety, and environmental concerns in a circular economy context by utilizing biodegradable polymers from agro-food waste residues (Guillard et al., 2018). Mathematical modeling of mass transfer and reactions in food/packaging systems is emerging as a valuable tool for designing and

validating new packaging solutions (Guillard et al., 2018).

MATERIALS AND METHODS

A thorough literature review was systematically carried out by sourcing peer-reviewed journal articles from a range of reputable scientific databases. The review process prioritized methodological rigor and relevance, focusing on studies that explored innovative packaging and labeling solutions specifically for ready-to-eat (RTE) meals.

Key selection criteria included research centred on antimicrobial packaging technologies, biodegradable material innovations, intelligent labeling systems, and strategies for extending product shelf life. Emphasis was placed on recent advancements and sustainability-oriented approaches, with particular attention to case studies and findings relevant to Romania's experience as part of the European Union.

The demand for ready-to-eat (RTE) meals has increased significantly due to changing consumer lifestyles. However, ensuring food safety, extending shelf life, and maintaining sustainability remain critical challenges. Modern packaging solutions integrate active components and intelligent labeling to address these concerns. This review highlights the latest advancements in packaging and labeling technologies and their implications for the food industry.

While looking for cutting-edge packaging methods the review compiles the latest and most advanced technologies and techniques used in packaging to improve the functionality, sustainability, and overall quality of packaging solutions. These methods go beyond traditional packaging and leverage innovation to meet the evolving demands of consumers, the environment, and the market, presented in Table 1.

Table 1. Cutting edges methods of packaging RTE-meals

Method	Scope	Effect
Active packaging	Involves incorporating materials or components into the packaging that interact with the contents to improve shelf life or preserve food quality and includes: - oxygen scavengers (absorb oxygen to prevent oxidation and spoilage); - moisture regulators (control moisture levels to prevent bacterial growth); - antimicrobial agents (release antimicrobial substances to prevent microbial growth).	- maintains product freshness; - improves shelf life; - helps reduce food waste.
Modified Atmosphere Packaging (MAP)	Changes the air inside the packaging using gases like nitrogen, oxygen, and carbon dioxide to slow down the deterioration of perishable goods. Commonly used for fresh produce, meats, and dairy products.	- slows microbial growth; - reduces oxidation; - preserves texture, color, and flavor of food.
Nanotechnology-based packaging	Uses nanotechnology to create thin, strong packaging materials with enhanced properties. Includes: - smart packaging (detects environmental changes like temperature or microbial contamination); - active coatings (antimicrobial surfaces).	- improved barrier properties (resistant to oxygen, moisture, and light); - extends shelf life.
Intelligent packaging	Integrates technology to provide real-time information about the product's condition inside the packaging, such as: - time-temperature indicators (TTIs) that change color to show exposure to improper temperatures; - freshness sensors that detect spoilage and alert consumers.	- enhances consumer confidence - reduces food waste; - provides accurate, real-time information.

Method	Scope	Effect
Sustainable packaging	Focuses on reducing environmental impact by using materials and technologies that are eco-friendly, such as: - minimalist packaging (reducing excess packaging to decrease resource consumption and waste).	- biodegradable materials (made from renewable sources like PLA); - reduces environmental impact; - edible packaging (can be consumed along with the product); - recyclable and reusable packaging.
3D Printing in packaging	Creates custom packaging solutions using 3D printing technology. Includes: - less material usage (optimizes packaging materials, reducing waste)	- on-demand production (reduces excess waste and storage costs); - customizable shapes.
Smart labels and QR codes	Enhances consumer engagement through technology like QR codes, providing access to product information, such as: - tracks product freshness (real-time data on product storage conditions); - enhances branding.	- provides detailed product information (origin, nutritional value, production processes).
Lightweight packaging	Focuses on reducing the weight of packaging materials. Includes: - eco-friendly alternatives (thinner plastics or paper that are easier to recycle or compost).	- reduces transportation costs (lower fuel consumption and carbon emissions); - lowers material costs.

(Adapted after Guillard V. et al., 2018, Janjarasskul T. et al., 2017)

Cutting-edge packaging methods are transforming the way we think about product preservation, sustainability, and consumer interaction. By using advanced materials and technologies like active packaging, intelligent labeling, nanotechnology, and sustainable alternatives, these innovations aim to not only enhance product quality and safety but also contribute to a more environmentally responsible future. The goal is to provide consumers with better, fresher products while reducing waste and improving the sustainability of packaging materials.

These innovations are reshaping industries and will continue to play a vital role in meeting the growing demands of modern consumers and the global environment.

RESULTS AND DISCUSSIONS

After careful consideration of all the collected data and good practices, the results related with innovative packaging and labeling solutions for preservation-free RTE-meals in the world, but also, some of them, with great implementation in Romania, are concluded in the following most efficient solutions:

1. Active and IP (intelligent packaging) technologies

Active packaging systems incorporate substances interacting with food or the surrounding environment to prolong shelf life. Examples include antimicrobial films (Fadiji et al., 2023), oxygen scavengers (Han et al., 2018), and ethylene absorbers (Janjarasskul, T. et al., 2017). Intelligent packaging, such as time-temperature indicators (Bumbudsanpharoke et al., 2022) and freshness sensors (Janjarasskul, Suppakul, 2017), helps consumers to monitor storage conditions, reducing food waste.

2. Sustainable and biodegradable packaging

With increasing environmental concerns, sustainable packaging solutions such as edible films (Trajkovska et al., 2021) and biodegradable polymers (Guillard et al., 2018) are gaining traction. These materials aim to replace traditional plastics while maintaining functional properties.

Sustainable packaging for RTE meals is crucial in addressing environmental concerns while meeting the growing consumer demand for healthier, more responsible options (Gutierrez et al., 2017).

The specialized literature offers more detailed exploration of how eco-friendly packaging being developed for RTE meals, such as:

2.1. ***Biodegradable and compostable packaging***

One of the most significant advancements in eco-friendly packaging is the use of biodegradable and compostable materials. For RTE meals, this can include:

- Plant-based polymers: Materials like polylactic acid (PLA), which are derived from renewable resources like corn starch or sugarcane, can be used to create containers, wraps, and trays for meals. PLA is compostable in industrial composting facilities and can break down without leaving harmful residues (Baneshi et al., 2024);
- Mushroom packaging: This innovative packaging material is made from mycelium (the root structure of mushrooms), which is biodegradable and compostable. It can be used as a protective layer for RTE meals, reducing plastic waste (Verma et al., 2023);
- Seaweed packaging: Some companies are exploring seaweed-based packaging, which is biodegradable and can even be edible in some cases. Seaweed has a minimal environmental footprint and grows quickly, making it a renewable resource for packaging (Kajla et al., 2024).

2.2. ***Edible packaging***

In some innovative RTE meal products, edible packaging is being developed to minimize waste. For example:

- Cereal and legume protein edible films: These can be used to wrap meals, and consumers can either consume the packaging with the meal or dispose of it in an environmentally friendly way (Linares-Castañeda et al., 2023);
- Gelatin and kitosan films: These materials can be used to encase food in a way that is both functional and edible, offering a sustainable solution to reduce the need for plastic packaging (Ștefănescu et al., 2022).

2.3. ***Recyclable materials***

For RTE meals, recyclable packaging is a priority in reducing the environmental impact of food packaging. Key trends include:

- Recyclable plastic trays and containers: companies are designing trays, bowls, and containers from recyclable plastics like polyethylene (PE) or polypropylene (PP), which can be processed and reused in the recycling system. Accordingly, with the life cycle assessment of food, the recyclable plastic trays and containers increased with 30% reaching 70% when properly manufactured and manipulated (Molina-Besch et al., 2018);
- Glass containers: while heavier and more costly, glass is an excellent recyclable material. Some RTE meals, especially those in higher-end or premium markets, use glass jars or containers that are fully recyclable and reusable (futuremarketinsights, 2025).

2.4. ***Minimalist and reduced packaging***

Sustainability in packaging often means reducing the amount of material used overall. In the case of RTE meals, this can involve:

- Streamlined packaging designs: fewer layers or unnecessary components, such as plastic wraps or excessive labels, to reduce waste and lower the carbon footprint of production and shipping;
- Smaller portion sizes: in some cases, reducing portion sizes for certain products can lead to smaller, more efficient packaging, reducing material usage;
- Slimmer packaging: Using thinner layers or smaller containers to hold the same amount of food reduces the amount of material used, which also lowers waste.

2.5. ***Reusability and refill options***

Another way to improve sustainability in RTE meal packaging is by focusing on reusable packaging systems:

- Reusable containers: Instead of single-use packaging, companies can offer RTE meals in containers designed to be reused. Consumers can bring these containers back for refills or to store other food items, cutting down on waste.
- Refill stations: Some innovative systems allow customers to buy RTE meals in reusable containers that can be refilled at designated stores, much like refillable beverage containers. This not only reduces single-use packaging but also encourages sustainable consumer behavior.

2.6. *Smart packaging for reduced waste*

Smart packaging plays an essential role in reducing food waste by helping consumers understand the product's condition and encouraging proper use. For RTE meals, these smart technologies can be used to:

- Time-temperature indicators (TTIs): These indicators change color based on exposure to temperature, showing whether a meal has been stored improperly. This can help consumers avoid consuming spoiled food, reducing food waste (Waldhans et al., 2025);
- Freshness sensors: Some packages contain sensors that monitor the freshness of the meal inside, signaling when the product is still at its best or when it may have gone past its optimal freshness.

2.7. *Sustainably sourced materials*

Using materials that are sustainably sourced is a key component of eco-friendly packaging for RTE meals, such as:

- Recycled paper and cardboard: Packaging made from recycled paper or cardboard is a more eco-friendly option compared to virgin paper materials. These can be used for outer packaging or as a protective layer inside containers.
- Sustainably sourced pulp: Packaging made from tree-free, sustainably sourced pulp (such as agricultural waste, bamboo, or sugarcane bagasse) reduces the reliance on virgin wood pulp and helps protect forests.
- Forest Stewardship Council (FSC) certified paper: For any paper or cardboard packaging, choosing FSC-certified materials ensures that they come from responsibly managed forests, supporting biodiversity and sustainable forestry practices.

2.8. *Eco-friendly inks and labels*

In addition to sustainable materials, packaging for RTE meals also needs to consider the inks and labels used. For an eco-friendly approach:

- Water-based inks: These inks are made without petroleum-based chemicals and can be used for printing on packaging materials like paper, cardboard, or bioplastics. They are less harmful to the environment compared to traditional oil-based inks;
- Plant-based labels: Labels made from plant-based materials like soy or corn starch can reduce environmental impact, especially when they are designed to be biodegradable;
- Minimalist labeling: Reducing the size of labels or printing information directly on the packaging material can reduce waste and the need for additional packaging layers.

2.9. *Carbon footprint reduction in packaging production*

Reducing the carbon footprint of packaging production is another way to make RTE meal packaging more eco-friendly. Innovations in the manufacturing process, such as:

- Energy-efficient production methods: Using renewable energy sources in the production of packaging materials can significantly reduce the carbon footprint of the packaging process;
- Local production: Manufacturing packaging materials closer to where RTE meals are produced can help reduce transportation emissions, thus decreasing the overall environmental impact.

3. Shelf-life extension strategies for RTE meals

Recent studies emphasize the role of coatings and modified atmosphere packaging in extending food shelf life. The use of turmeric and red ginger coatings (Dharma, 2023) and the impact of packaging on ready-to-eat meals (Clodoveo et al., 2021) demonstrate effective strategies for prolonging product freshness. Modified atmosphere packaging (MAP) is particularly useful in preventing microbial growth and oxidation, thereby preserving food quality (Kontominas et al., 2021).

4. Study case Romania – good practice in extending the shelf life of RTE meals

Several noteworthy practices and innovations have been identified in Romania, as documented and analyzed in Table 2.

Future packaging methods with increasing interest from the Romanian RTE producers are presented in Table 3.

Table 2. Modern RTE packaging in Romania

Practice/Technology	Description	Reference
Micvac Technology	Caroli Foods Group introduced a range of chilled ready meals under their Maestro brand using Micvac's in-pack cooking and pasteurization technology. This method allows meals to retain flavor and nutrition for up to 60 days without additives or preservatives.	Caroli Foods Group launches range of ready meals using Micvac technology (www.foodanddrinktechnology.com)
Biobased and Biodegradable Packaging	BFG Packaging produces 100% biobased and biodegradable single-use rigid takeaway packaging made entirely from FSC-certified wood fiber. This eco-friendly packaging is oil and water-resistant, heat-retaining, and heat-sealable, aligning with EU regulations promoting ecological packaging.	Biobased and biodegradable single use rigid takeaway packaging now produced in Romania (www.bfgpackaging.com)
Aseptic Carton Packaging	Tetra Pak Romania offers aseptic carton packages that protect perishable foods, such as milk and juices, for up to 12 months without refrigeration or preservatives. The multi-layered design safeguards contents from light, oxygen, and moisture, preserving quality and safety.	Food protection by Tetra Pak Romania (www.tetrapak.com)
Vacuum Skin Packaging (VSP)	VSP technology involves sealing the product with a transparent film that conforms to its shape, removing air and extending shelf life. This method enhances product presentation and freshness, making it suitable for various food items, including RTE meals. However, this method is one of the oldest methods of packaging with the purpose of enhancing shelf life of RTE meals.	How Vacuum Skin Packaging Can Increase Your Product Shelf Life & Sales (www.zonesone.com/ro)
Tetra Pak's eBeam Technology at Simultan	In January 2025, Tetra Pak installed its advanced eBeam technology at Simultan, Romania's largest locally-owned dairy processor. This innovative packaging line utilizes electron beam technology for sterilization, reducing environmental impact and enhancing the shelf-life of dairy products.	packagingreporter.com/food-beverage/tetra-pak-and-simultan
CHEP's Circular Logistics Solutions	CHEP Romania has implemented circular logistics solutions by providing reusable pallets for product transportation. This approach reduces waste and carbon footprint, contributing to a more sustainable supply chain in the FMCG and retail sectors.	FMCG and Retail Sectors drive Romania's transition to the Circular Economy (www.chep.com/ro/en/fmcg-and-retail-sectors-drive-romanias-transition-circular-economy)
Tetra Recart® Packaging	Tetra Pak Romania offers Tetra Recart®, an innovative carton-based packaging solution suitable for shelf-stable foods like vegetables, soups, and ready meals. This packaging extends shelf life without refrigeration and has a lower climate impact compared to traditional packaging methods.	Food packaging by Tetra Pak Romania (www.tetrapak.com)

Table 3. Emerging packaging methods for enhancing RTE meals' shelf-life

Method	Functionality	References
Active Packaging Using Agri-Food Waste	- active packaging incorporating activated carbon derived from agri-food waste. This packaging effectively adsorbs ethylene, delaying the ripening of fruits and vegetables and extending shelf-life. .	Fadiji et al., 2022
Copper-Infused Packaging	- packaging method that incorporates copper microparticles, exhibiting biocidal properties against microorganisms. This technology extends the shelf-life of various food products by up to 30 days.	Gopinath et al., 2024, https://packagingeurope.com/news/copper-infused-masterbatch
Microencapsulation of Extra Virgin Olive Oil (EVOO)	- microencapsulation of EVOO in dietary fiber, resulting in breaded foods with up to 90% less fat and an extended shelf-life of up to three weeks. This technology offers a healthier alternative to traditional frying methods and prolongs product freshness.	Calvo et al., 2012; Başyiğit et al., 2021

Innovative packaging and labeling solutions for RTE meals present significant benefits, including enhanced food safety, waste reduction, and improved consumer transparency. However, challenges such as cost-effectiveness, regulatory compliance, and material compatibility remain. Future research should focus on scaling biodegradable alternatives and integrating digital tracking technologies to improve supply chain efficiency.

Market research indicates a growing consumer willingness to transition from traditional dining habits to a ready-to-eat (RTE) meal model by 2035. This projected shift is supported by the factors outlined in Table 4, which highlight key drivers behind this behavioral change.

Table 4. Market shift 2025 to 2035 (futuremarketinginsights.com)

Factors	Behavioral change
Regulatory Landscape	Regulatory frameworks today target clean-label foods, reduced sodium and sugar, and natural preservatives. Governments worldwide promote green packaging and heightened cold chain regulation to minimize food wastage.
Technological Advancements	AI-facilitated supply chain optimization guards against minimal waste and precise demand forecasting. Smart packaging with shelf-life markers and blockchain traceability enhances food safety and transparency in RTE foods.
Consumer Preferences	Health-conscious consumers drive demand for plant-based, organically grown, and nutritionally balanced RTE food. Growth in allergen-free and keto, paleo, and high-protein meal opportunities meets niche nutritional needs.
Ingredient Sourcing & Sustainability	Growing global acceptance of regenerative farming and carbon-zero ingredient sourcing. Companies use alternative proteins, such as lab-cultured meat and plant protein, to reduce their environmental impact.
Packaging Innovations	Smart, edible, and biodegradable packaging solutions dominate, with QR codes providing in-depth sourcing and nutritional data to consumers.
E-commerce & Retail Dynamics	Direct-to-consumer (DTC) players thrive on customized meal plans. AI-powered recommendations and fast-delivery networks achieve maximum convenience and product availability.
Regional Market Trends	Regional taste creation and personalization drive market growth. Emerging markets witness a boom in fortified and nutrient-fortified RTE meals to combat malnutrition.
Market Growth Drivers	Plant-based protein innovation, sustainable production, and minimally processed, nutrient-rich RTE meal demand drive long-term growth. Flexitarian and personalized nutrition trends also drive growth.

CONCLUSIONS

The advancements in packaging and labeling solutions offer promising alternatives to conventional methods for RTE meals. Their role in food safety, quality maintenance, and sustainability is crucial in addressing global food preservation challenges. Continued research and technological integration will drive further improvements in this field.

The RTE meals industry in Romania faces a dual challenge: extending shelf life while promoting sustainability. Companies involved in the RTE meals industry in Romania are actively implementing innovative packaging solutions to enhance food preservation without compromising quality. However, these advancements come with challenges, including higher production costs, consumer acceptance of new technologies, and regulatory compliance with EU sustainability goals.

To remain competitive, the Romanian RTE meal producers must strike a balance between extending shelf life and reducing environmental impact. Sustainable solutions such as biodegradable materials, active packaging, and circular economy models (e.g., reusable packaging) are gradually being adopted. Despite the progress, more investment in research, collaboration between food technologists and packaging specialists, and consumer education will be key to ensuring long-term success.

The future of the Romanian RTE meals industry depends on innovative, cost-effective, and sustainable packaging solutions that meet both market demand and environmental standards, paving the way for a more resilient and eco-friendly food sector.

In response to the growing consumer demand for clean-label, preservative-free food products, the integration of advanced packaging

technologies emerges as a critical component in ensuring the safety, quality, and regulatory compliance of ready-to-eat (RTE) meals. The adoption of these innovations not only aligns with evolving environmental standards but also supports the development of more sustainable food preservation practices. Continued interdisciplinary research and collaboration within the industry will be instrumental in advancing the implementation and optimization of these technologies.

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EMERGING TRENDS IN FOOD WASTE REDUCTION AND RESIDUE VALORIZATION: ADVANCING SUSTAINABILITY IN THE FOOD SERVICE INDUSTRY - STUDY CASE IN ROMANIA

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Abstract

The food service industry faces a growing challenge of managing food wastage while transitioning toward sustainable practices. This research explores emerging trends in food residue valorisation, focusing on innovative techniques for transforming food waste into valuable by-products such as bioenergy, animal feed, and bio-based packaging materials. Key advancements in bioprocessing technologies, including anaerobic digestion, composting, and enzymatic treatments, are evaluated for their efficiency and scalability. Additionally, this study examines the role of digital tools, such as AI-driven waste tracking and optimization systems, in minimizing waste generation. The integration of circular economy principles within the food service sector is highlighted as a pivotal strategy to address environmental, economic, and social sustainability. By identifying and analysing these new trends, the research provides actionable insights for stakeholders aiming to reduce waste and valorise residues effectively as well in agrifood industry and in food service industry, particularly in Romania's case.

Key words*: anaerobic digestion, circular economy, zero waste, sustainable food service, upcycling.*

INTRODUCTION

Food waste is one of the most pressing global environmental issues. It occurs across all stages of the food supply chain, but a significant portion is generated in the food service industry, from restaurants to ready-to-eat (RTE) meals. This waste contributes to increased greenhouse gas emissions, depletion of natural resources, and the loss of valuable nutrients. In recent years, the food service sector has shifted towards more sustainable practices, with a focus on waste reduction and residue valorisation (Arancon et al., 2013; Berenguer et al., 2022)

Food residue valorisation refers to the process of converting waste by-products into valuable materials, biofuels (Castro-Muñoz et al., 2021), or functional food ingredients. Through such practices, the food industry not only reduces waste but also contributes to environmental sustainability and economic growth. Recent innovations in packaging technologies have proven to be pivotal in extending the shelf life of food products and reducing waste in the process.

The objective of this review is to examine the emerging trends in food waste reduction, with a particular focus on the valorisation of food residues and advances in packaging technologies. Through an extensive analysis of the literature, it is explored how these innovations are advancing sustainability and reducing environmental impacts in the food service industry.

Recent research highlights emerging trends in food waste reduction and valorisation within the food service industry. Innovative waste management practices are being explored to address sustainability challenges, with implementation varying based on management's perspectives and goals (Martín-Ríos et al., 2018). The circular economy concept is gaining traction, emphasizing environmental protection and economic development through efficient utilization of food industry waste and by-products (Sharma et al., 2021). Encapsulation techniques for bioactive compounds extracted from food waste show promise, though further research on regulatory and toxicity considerations is needed (Borah et al., 2023). Waste valorisation strategies are being

developed to produce chemicals, materials, and fuels sustainably, moving beyond traditional disposal methods like landfills and incineration (Arancon et al., 2013). These approaches include biorefinery concepts for producing valuable products such as succinic acid and bioplastics from bakery waste (Arancon et al., 2013; Burange et al., 2016). The focus is shifting towards higher-value, marketable products from second-generation waste valorisation strategies (Burange et al., 2016). Other good practices in the Netherlands (Verkleij et al., 2019) underline the importance of promote the effort of collaborative teamwork to reduce with 50% the food waste.

MATERIALS AND METHODS

This review analyses various academic published articles, accessed via Science Direct, Web of Science (Enformation platform), Google Scholar, books, and case studies focused on food waste management, residue valorisation, and sustainable packaging. A systematic review methodology was employed to synthesize the key findings from the most recent research.

The literature was analysed using the following criteria, words searching and issues: "*Emerging technologies for food waste valorisation: Trends in turning food residues into valuable by-products*"; "*Innovations in sustainable packaging: Focus on active, intelligent, and biodegradable packaging*"; "*Environmental and economic impacts: Considerations of the sustainability of these technologies and their effect on reducing waste and conserving resources*"; "*How does the Romanian government has implemented measures to promote sustainability by reducing food waste*"; "*Actions to reduce food waste in the Romanian food service industry*".

RESULTS AND DISCUSSIONS

1. Active packaging and shelf-life extension

Active packaging is a technology that integrates materials or components into packaging systems that actively interact with the contents to improve food quality and shelf life. As discussed by Prasad & Kochhar (2014), active packaging includes components like oxygen scavengers, moisture regulators, and antimicrobial agents, which can significantly extend shelf life and reduce food waste. These packaging systems are designed to react with environmental factors (e.g., oxygen, moisture) to maintain the food's freshness.

For example, Dharma (2023) explored the use of turmeric and red ginger as natural antimicrobial film coatings for RTE meat products. This active packaging method extends the shelf life by preventing microbial growth, which is a significant contributor to food spoilage.

The most common active packaging technologies and their effect on food are presented in Table 1.

Table 1. Active packaging technologies and their effects (adapted from Prasad & Kochhar, 2014; Dharma, 2023)

Active packaging type	Function	Effect on food
Oxygen Scavengers	Absorb oxygen from the package	Reduces oxidation and spoilage
Moisture Regulators	Control moisture inside the package	Prevents bacterial growth and mold
Antimicrobial Agents	Release antimicrobial substances	Inhibit the growth of bacteria, molds, fungi

2. Modified Atmosphere Packaging (MAP)

Modified Atmosphere Packaging (MAP) involves altering the composition of gases inside the package to slow down the deterioration of food products. Berenguer et al. (2022) noted that MAP is commonly used for fresh produce, meats, and dairy products. By adjusting the levels of oxygen, nitrogen, and carbon dioxide, MAP creates an optimal environment that:

- Slows microbial growth;
- Reduces oxidation;
- Preserves food texture, colour, and flavour.

In Figure 1, there is a presentation of different gaseous composition in MAP for different types of food.

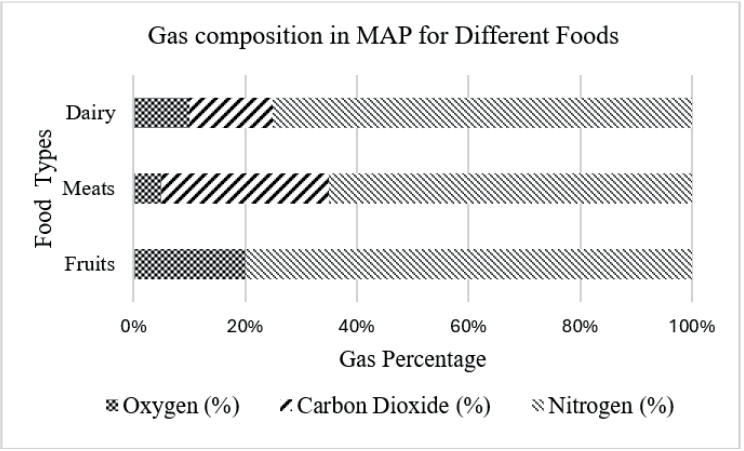


Figure 1. Evolution of Average Milk Yield

It can be observed (Figure 1) that for dairy, the composition of MAP is High in N_2 (inert gas to prevent oxidation), low CO_2 (minimal antimicrobial need); for meats: High CO_2 (extends shelf life by inhibiting bacteria), moderate O_2 (preserves red colour) and for fruits: Balanced O_2/CO_2 (slows ripening; exact ratios vary by fruit type).

3. Smart and intelligent packaging systems

Intelligent packaging goes beyond simple preservation by integrating technology that monitors and reports the status of the food. Smart labels, time-temperature indicators (TTIs), and freshness sensors provide real-time data to consumers, helping them make informed decisions about food quality and safety. As discussed by Biji et al. (2015), these technologies enhance consumer confidence and reduce food waste by ensuring products are consumed within their optimal time frame.

For instance, Corradini (2018) reviewed time-temperature indicators (TTIs), which change colour to indicate whether a product has been exposed to improper temperatures during storage or transportation. This type of intelligent packaging is crucial for perishable foods, particularly in the meat and dairy industries.

4. Valorisation of food waste and by-products

Food industry waste, including peels, seeds, and shells, has enormous potential for valorisation (Ribeiro, 2022; Roy et al., 2023). By converting these residues into valuable products such as biofuels, bioplastics, and functional food ingredients, the food service industry can minimize waste and contribute to a circular economy. Lin et al. (2014) and Ravindran et al. (2016) highlighted various methods for transforming food waste into value-added products.

- Bio-based materials: Food residues such as fruit peels can be processed into bioplastics and bio-based packaging materials or chemical materials and biofuels (Ong et al., 2018; Nayak et al., 2023).
- Bioactive compounds: The extraction of bioactive compounds from food residues (such as phytonutrients) can lead to new functional food ingredients (Luca, 2022).

Different methods for transforming food waste into value-added products are presented in Table 2.

Table 2. Valorisation of Food Industry Wastes (adapted from Ravindran et al., 2016; Borah et al., 2023)

Food waste type	Valorisation product	Applications
Fruit peels	Bioplastics, bioactive compounds	Packaging, functional foods
Coffee grounds	Biofuels, nutraceuticals	Energy production, health supplements
Vegetable trimmings	Animal feed, fertilizers	Agriculture, sustainability

There is also valuable research in Romania related with high utilization of by-products, such as pomace from different natural juices made from carrots, apple, pear also. In Table 3 there is a non-exhaustive list of the Romanian researcher of valorisation products in food industry.

Also, Vodnar et al. (2015) has been developed an edible film with antimicrobial properties to increase the pork meat preservation under refrigerated condition; Pop et al. (2013) have developed different antibacterial extracts from plants, underlying the focus on sustainability and reducing the food waste in Romania. Szabo et al. (2018) have used the tomato by-products to obtain different valuable nutritive products. Socaci et al. (2018) investigated the influence of the extraction solvent on phenolic content, antioxidant, antimicrobial and antimutagenic activities of brewers' spent grain.

Utilisation of by-products for valorisation were investigated to reduce food waste. The obtaining of bioactive compounds from red beetroot peel powder were studied by Constantin et al. (2025) with the developing of merengue products, by Stoica et al. (2024) for whey-fruit based beverage development and apple for potentially functional apple snacks by Stan et al. (2025). Despite the un-commercial shape, apples can be valorised in crispy snacks reducing with 80% the food waste. In Table 3 there are mentioned some important contribution.

Table 3. Ways of valorisation of some vegetable by-products into sustainable products

By-products type	Valorisation product	Applications
Red beetroot peel powder	Bioactive compounds, natural dye and food preservative	Meringue Products (Constantin et al., 2025)
Red beet peel	Phenolics and betalains compounds	Whey-fruit-based beverage (Stoica et al., 2024)
Apple snacks	Apples	Potentially Functional Apple Snacks (Stan et al., 2025)

5. Sustainable and edible packaging

Sustainable packaging materials, such as biodegradable polymers and edible films, are essential for reducing plastic waste in the food industry. Trajkovska Petkoska et al. (2021) reviewed several sustainable packaging materials, including those made from plant-based sources like PLA (polylactic acid) and starch-based films. Furthermore, edible packaging made from natural ingredients such as seaweed, rice, and proteins offers a novel approach to reducing waste.

For example, Gil & Rudy (2023) highlighted how edible packaging made from fish gelatine could be used for meat products, offering both environmental benefits and added functionality to the food packaging industry.

6. 3D Printing in packaging

3D printing technology is revolutionizing packaging by enabling the creation of customized packaging designs. As Bumbudsanpharoke & Ko (2022) discussed, 3D printing allows for on-demand production, reducing waste in packaging production and enabling the creation of packaging with optimized shapes to minimize material usage. The use of this technology contributes to sustainability by reducing the environmental impact of overproduction and excess packaging.



Figure 2. 3D Printed Packaging for Customization (source: www.packnode.org)

The rise of 3D printing technology is revolutionizing the packaging industry, enabling unparalleled customization, faster prototyping, and more sustainable production

processes. This innovative method is reshaping how packaging is designed and manufactured. 3D printing is used to create prototypes, molds, and even fully functional packaging components. It allows designers to test and iterate quickly, reducing time-to-market for new products. For customized packaging, 3D printing enables intricate designs tailored to specific products or brands.

Key advantages of 3D printing technology of packages:

- Flexibility: Adapts to small-batch production, ideal for limited editions or unique product lines.
- Sustainability: Reduces material waste by producing only what is needed.
- Speed: Accelerates the prototyping process, allowing for rapid innovation.

Despite its benefits, 3D printing in packaging faces challenges like high initial costs and limited scalability for large-scale production. However, advancements in materials and printing technology are addressing these barriers, making it more accessible to businesses of all sizes.

As 3D printing continues to evolve, it is set to become a cornerstone of the packaging industry, offering solutions that are not only innovative but also aligned with the demands of modern consumers and sustainability goals.

7. How has the Romanian government implemented measures to promote sustainability by reducing food waste

As a member of the European Union, Romania has demonstrated a strong commitment to implementing significant regulatory measures in recent years to align with the objectives set by the European Parliament. These measures specifically aim to mitigate food waste, targeting a 10% reduction in household food waste and up to a 30% reduction in the food industry, including the food service sector. Among the important undertakings are:

1. Legislation to Combat Food Waste: In 2016, Romania enacted Law no. 217/2016, obligating entities in the agri-food sector to adopt measures preventing food waste. These measures encompass actions such as selling products nearing their expiration dates at reduced prices, donating near-expiry food to registered organizations, and repurposing unfit food for animal consumption, composting, or biogas production. Non-compliance with this law can result in fines ranging from RON 1,000 to RON 10,000, depending on the size of the enterprise (cms-lawnow.com);
2. Amendments to Strengthen Food Waste Reduction: In March 2024, Law no. 49/2024 was enacted to further enhance efforts against food waste. This law mandates that businesses such as shops, restaurants, and canteens implement at least two measures to reduce food waste before discarding food. These measures include reducing prices on products nearing expiration, donating food, or repurposing it for animal feed. Additionally, the law simplifies procedures for non-governmental organizations to receive non-perishable food and establishes a national platform, managed by the Ministry of Agriculture, for reporting food waste data (foodcomplianceinternational.com);
3. National Strategy for Food Waste Reduction: The government is developing a comprehensive National Strategy for the Prevention and Reduction of Food Waste. This strategy aims to outline actions to prevent and reduce food waste, set quantifiable targets, promote anti-waste measures, assign responsibilities, and allocate necessary financial resources. The strategy is to be updated every five years and presented to the Romanian Parliament before adoption (agroberichtenbuitenland.nl).
4. Public Awareness and Education Campaigns: The Ministry of Agriculture and Rural Development, in collaboration with the Department for Sustainable Development, has initiated campaigns to raise public awareness about food waste. These efforts include creating impactful anti-waste messages and translating educational materials from the Food and Agriculture Organisation (FAO) for distribution in schools across Romania (ec.europa.eu).

Through these comprehensive measures, the Romanian government aims to significantly

reduce food waste, promoting sustainability and environmental responsibility nationwide.
Actions to reduce food waste in the Romanian food service industry have been guided by national sustainability goals and aligned with EU regulations promoting circular economy practices, presented in Table 4.

Table 4. Good practice in food waste management and sustainability in the Romanian food service industry

Action	Example	Impact
Waste Reduction in Restaurants	Cluj, Timisoara and Bucharest's restaurants are offering smaller portion sizes to minimize leftovers, encouraging customers to only take what they can finish. Additionally, restaurants like **Caru' cu Bere** in Bucharest are part of food waste reduction initiatives by working with local charities to donate excess food at the end of the day.	Up to 30% of food waste reduced in one year (2021-2022, according with Ghenea, 2022)
Repurposing Food Scraps	Some restaurants and food service businesses in Romania are repurposing food scraps. For instance, vegetable peels, stems, and other by-products from meal preparation are used to make broths or soups, which are then served to customers as a sustainable offering. Additionally, they are turning stale bread into croutons or breadcrumbs, and fruit scraps are used to make juices or preserves.	The purchasing cost of ingredients decreased up to 30% while the enhancing of the taste increased with 50% without makeover with taste-enhancers. (Săftoiu et al., 2021)
Zero-Waste Kitchens	Careful planning to reduce food waste, reusing ingredients creatively, and composting organic waste. Some chefs have started using techniques like fermentation or pickling to preserve food by-products or underutilized ingredients, which also results in innovative new dishes	Decreasing of the food waste by careful planning of the menu and purchasing ingredients with up to 20% in one year time (Radu & Mihailescu, 2023)
Sustainability Partnerships	Some food service chains and restaurants in Romania partner with food recovery organizations, such as **Feed Forward**, which helps reduce food waste by redistributing excess food to those in need. These partnerships are essential in supporting the circular economy and reducing food waste in the food service industry.	Reducing food waste by sustainability partnership reached 20% in the involved restaurants (Andrei I., 2020)
Food Waste Awareness Campaigns	**„Sunt un Restaurant Sustenabil"** ("**I am a Sustainable Restaurant"**) is an initiative in Romania aimed at raising awareness and encouraging food service businesses to adopt sustainable practices. This initiative focuses on reducing food waste, offering portion control options, and repurposing food by-products. It also promotes partnerships with local NGOs to donate leftover food to people in need.	Up to 30% of food waste was reduced by activating awareness campaigns in 2020. However, the Covid19 impacted severely the food service industry, and some data could be overrated (Hossu et al., 2021)
Food Waste Reduction Apps	In cities like Bucharest, several food service businesses have begun using apps like **Too Good to Go** or **Savery – stop food waste** which allows them to sell surplus food at discounted prices, helping to reduce waste and provide affordable meals to customers. This is particularly useful for cafés, bakeries, and small food outlets that often have excess food at the end of the day	Using the apps enable the users to find ways not to throw away the extra-food but to sell with discounted price (up to 35% for each order, or half price for the second order). Mainly pizza, shawarma, poke-bowl or noodles food service units are using these apps with a reduction of food waste reaching 30% yearly. (Constantin, I., 2022)
Biodegradable Packaging and reusables containers	Several food service providers are shifting to biodegradable or compostable packaging to reduce waste. This includes adopting eco-friendly takeaway containers made from plant-based materials, helping reduce the environmental impact of disposable packaging in the food service industry. Also, on European Union recommendation, the reusable containers will be warmly supported starting with 2027 in food service industry, especially in take-a-way system or home-delivery system based on abonnement.	By shifting to biodegradable packaging and reusable containers the food service industry in Romania can mitigate the food-waste with up to 10% (Popescu, C., 2020)

CONCLUSIONS

Emerging trends in food waste reduction and residue valorisation hold great promise for transforming the food service industry towards greater sustainability. The integration of active, intelligent, and biodegradable packaging systems plays a crucial role in extending the shelf life of food and reducing spoilage. Furthermore, the valorisation of food industry by-products into valuable resources supports the transition to a more circular economy, where food residues are converted into biofuels, bio-based materials, and functional food ingredients.

These advancements not only address the growing concerns over food waste but also contribute to significant environmental and economic benefits. Future research should continue to explore ways to improve the efficiency of these technologies, focusing on scalability, cost-effectiveness, and minimizing the overall environmental footprint of food production and packaging.

By embracing these innovative technologies, the food service industry can make significant strides toward sustainability, ensuring a more efficient, waste-free, and eco-friendly future.

The food service industry in Romania is increasingly recognizing the critical importance of sustainability and food waste reduction in aligning with both local and European Union sustainability targets. Numerous initiatives within the sector demonstrate a clear commitment to adopting circular economy principles and minimizing environmental impact. From repurposing food scraps into value-added products to reducing portion sizes and implementing zero-waste kitchen practices, the sector is actively engaged in fostering sustainable practices.

Collaborations with food recovery organizations, the adoption of digital tools such as food waste reduction apps, and the transition to biodegradable packaging reflect the growing trend toward environmental responsibility.

Moreover, Romania's food service sector is also advancing the idea of creating more efficient food systems through partnerships and technological innovations, contributing to the reduction of food waste. While challenges remain in ensuring widespread adoption of these practices, the sector's ongoing efforts illustrate a significant commitment to reaching EU sustainability targets and reducing its ecological footprint.

In conclusion, Romania's food service industry has made notable strides in mitigating food waste, but continued investment in education, collaboration, and innovative solutions will be essential in achieving long-term sustainability goals. Through these combined efforts, the sector can play a vital role in building a more sustainable and resource-efficient food system.

Particularly, at the Faculty of Animal Productions Engineering and Management, the Science in Gastronomy licence program deliver an exhaustive Sustainable gastronomy program, aiming to create awareness on reducing the food waste by taking the appropriate measures.

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THE IMPACT OF USING OLEOGEL AND BUCKWHEAT FLOUR ON THE PROPERTIES OF SEMI-SMOKED SAUSAGES

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Abstract

Semi-smoked sausages are a valued food product in Romania, but their high fat content raises significant public health concerns. This study proposes an innovative solution by replacing pork fat with oleogel and adding buckwheat flour, aiming to improve the physicochemical, organoleptic, technological, and functional properties of the product, thus offering a healthier and nutritionally balanced alternative.Oleogel, used as a substitute for animal fat, represents a modern alternative with the potential to reduce the saturated fat content in meat products. Through its structure, oleogel ensures a texture similar to traditional fat, contributing to the maintenance of desired organoleptic properties, such as juiciness and aroma. Additionally, the use of buckwheat flour adds functional benefits due to its rich content of fiber, proteins, and bioactive compounds with antioxidant properties. This combination promises an improvement not only in nutritional value but also in the stability and safety of the product. The study analyzes the impact of these changes on the main characteristics of semi-smoked sausages, such as texture, color, aroma, and technological behavior during processing and storage. The obtained results could contribute to the development of healthier meat products, tailored to current consumer demands, without compromising their quality or acceptability. This approach opens new perspectives in the food industry, promoting innovation and sustainability in food production.

Key words*: buckwheat flour, nutritional value, oleo gel, organoleptic properties, semi-smoked sausages.*

INTRODUCTION

It is essential or the global population to have access to food proteins that are both sustainable and healthy, such as those from plant-based raw materials (Sharma et al., 2016). For this reason, plant protein sources could represent a viable solution to feed a growing population, provided they meet amino acid requirements and are organoleptically acceptable (Neacsu et al., 2022). Ensuring access to food is an important global issue at all stages of human society development (Ghendov-Moșanu, 2021). According to specialists from the World Health Organization, human well-being is influenced 50% by each person's lifestyle, 20% by environmental conditions, another 20% by hereditary factors, and only 10% by medical services, which underscores the importance of individual choices, especially regarding nutrition (Popkin et al., 2012).

Currently, food products are created using advanced technologies that allow them to have a long shelf life and attractive sensory characteristics (Inetianbor et al., 2015).

The modification of food composition by adding considerable amounts of plant proteins can significantly influence the specific biochemical balance of a consumer's body (Juvan et al., 2005).

In this context, there is an increased need to develop functional food products (Lisitsyn et al., 2018). If a classification is pursued based on the type of food (cereals, meat products, pastries, etc.), the diseases they can prevent, or improve (such as cancer or diabetes), the physiological effects (for example, antitumor activity, digestion, immunity), and the type of biologically active compounds (antioxidants, vitamins, lipids, etc.), we can achieve a dietary balance among the population (Wieslander, 2020; Ianiţchi et al., 2024).

The use of plant-based raw materials in the production process of meat products represents a necessary and current trend (Prosekov, 2014). These raw materials are used to create functional products. In this category of products, dietary, therapeutic, and preventive options are included, intended for both children and the elderly (Satija et al., 2016).

Combining meat ingredients with plant-based ones contributes to increasing the nutritional and biological value of meat products, thus reducing losses during thermal processing (Prosekov et al., 2020). The introduction of plant raw materials into meat product recipes helps improve their physical, chemical, functional, technological, and organoleptic characteristics (Bronnikova et al., 2018).

In Romania, sausages are highly appreciated, being predominantly included in traditional diets. However, a concerning aspect for consumer health is the high content of saturated fatty acids. The long-term impact on health manifests through the increased incidence of cardiovascular diseases, chronic conditions, and obesity (Tilman et al., 2014). The purpose of this paper is to present the importance of protein additives in meat products, as the interest in a balanced and healthy diet has increased among the population.

Replacing pork fat with oleo gel and adding buckwheat flour, with the aim of improving the physicochemical, organoleptic, technological, and functional properties of the product, offers a healthier and nutritionally balanced alternative (Dinu et al., 2017).

MATERIALS AND METHODS

For the completion of this work, academic articles, specialized books, and official reports published between 1990 and 2024 were consulted. These resources were available on websites managed by renowned institutions as well as on search engines such as Google Scholar, PubMed, and Scopus.

The keywords used in the search included: "semi-smoked sausages", "oleo gel", "buckwheat flour", "organoleptic properties", and "nutritional value".

The selection of works was based on their relevance to the proposed topic, considering both experimental studies and synthesis articles. Materials that analysed both the chemical compounds of protein additives (buckwheat and oleo gel) and the importance of their use in meat products were included. The extracted information was then grouped and synthesized into the text, with the aim of providing a clear and accessible overview of the main conclusions from the specialized literature.

RESULTS AND DISCUSSIONS

Buckwheat: chemical composition

Buckwheat is a member of the Polygonaceous family, specifically the genus Fagopyrum (Sangma et al., 2010). Cymosum and urophyllum are its two monophyletic groups (Luthar et al., 2021).

A gluten-free crop, buckwheat has a lot of potential as a cereal alternative. The concentrations of mineral elements in two species of buckwheat, such as Tartary buckwheat (*Fagopyrum tataricum* Gaertn) and common buckwheat (*Fagopyrum esculentum* Moench), are found to be impacted by the way grains are processed into groats (using hydrothermal processing to remove the husk) and sprouts (7-day seedlings) (Zhao et al., 2023).

However, despite being a less common plant in agriculture, sarraceno trigo (*Fagopyrum esculentum* L.) has long been known for its health benefits. The alforfon semillas are dark-marred and triangular, measuring roughly 4-6 mm in length, 2.7-3.5 mm in width, and 2.5-3.3 mm in width. It is used in the form of flour for a variety of products (Zhang et al., 2021). The fact that buckwheat is a gluten-free grain is a significant feature that makes it appropriate for those who are gluten intolerant. With an absorption rate of up to 80-85%, buckwheat grains have the greatest protein content of any cereal (Bonafaccia et al., 2003). The chemical composition of buckwheat grains is presented in the Table 1. These proteins consist of globulins, glutelin, and important amino acids like cysteine, tryptophan, lysine, and threonine; buckwheat has 18 amino acids in total (Bonafaccia et al., 2003; Huda et al., 2021). Furthermore, rutin, a glucosidal flavonoid, helps preserve the flexibility of blood vessels, and buckwheat grains are high in antioxidants. About 25% of the carbohydrates in buckwheat are made up of starch (Woo et al., 2016).

Buckwheat grains are classified as pseudocereals with high nutritional value due to their protein content (Zhu, 2021).

Table 1. The chemical composition of buckwheat grains (Ghendov-Moșanu, 2021)

Indicators	Buckwheat
Water, %	11-12
Glucide, %	67-73
Lipid, %	2.3-3.6
Protide, %	12-18
Cellulose, %	7.3-10.9
Minerals, %	1.8-2.1

Although buckwheat has a relatively low protein content (common buckwheat: 10.6 g/100 g dry weight; tartary buckwheat: 10.3 g/100 g dry weight), it stands out due to its balanced amino acid composition, with significant levels of essential amino acids such as leucine and lysine (common buckwheat: 10.3 g/100 g protein; tartary buckwheat: 7.11 g and 6.18 g/100 g protein) (Bobkov, 2016).
Additionally, the high content of proteins, flavonoids, and trace elements in certain fractions of buckwheat suggests that they can be used in food products intended for special diets (Zhu, 2016). The nutritional value of buckwheat can be observed in Figure 1 and the chemical structures of the isolated components in Figure 2.



Figure 1. The nutritional value of buckwheat grains (S.R.L. Vitalcomus)

Buckwheat proteins can also contain selenium, an essential trace element for human nutrition (Skrabanja et al., 2004).

Oleogels: application

Regulations are becoming increasingly strict regarding the use of trans and saturated fatty acids (Igenbayev et al., 2022). Moreover, consumers are increasingly concerned about the negative effects of certain fats on health, leading to significant changes in the food industry (Badar, 2021).
Moreover, the environmental impact and health issues associated with the extensive use of palm oil, both in the food sector and in biodiesel production, have stimulated the development of innovative solutions for reformulating fat-containing products (Borșa et al., 2025).
Oleogelation has become one of the most widely used techniques for reducing or replacing unhealthy and controversial fats in foods (Liu et al., 2024). Various edible oleo gels are created through different methods. These are used in spreads, baked goods, confectionery, as well as in dairy and meat products (Perța-Crișan et al., 2023).
Oleo gels in food involve gelation mechanisms, while also discussing the properties of the new products (Kavya et al., 2024). Their use has led to the development of reformulated food products that are acceptable and have technological and rheological characteristics like those of traditional products, or even functionally improved. However, there is still a significant need to optimize the methods (Guo et al., 2022). Oleogels are obtained from oils derived from plant raw materials such as canola, sunflower, corn, soy, olives, flax, sea buckthorn, grapes, rice bran (Figure 3).

Oleogel - based meat products

Oleogels are widely used in the food industry for creating innovative products (Ye et al., 2019). We can identify oleogels in pastry and confectionery products (Patel et al., 2014; Mert et al., 2016), dairy products (Huang et al., 2018), and meat products (Pușcaș et al., 2020). Oleogels are used both for replacing trans and saturated fats and for their important role as carriers of water-insoluble substances, heat resistance, and stabilizers in products without emulsifiers (Pușcaș et al., 2020; Nacak et al., 2021). The characteristics of oleogels in meat products are mentioned in Table 2.

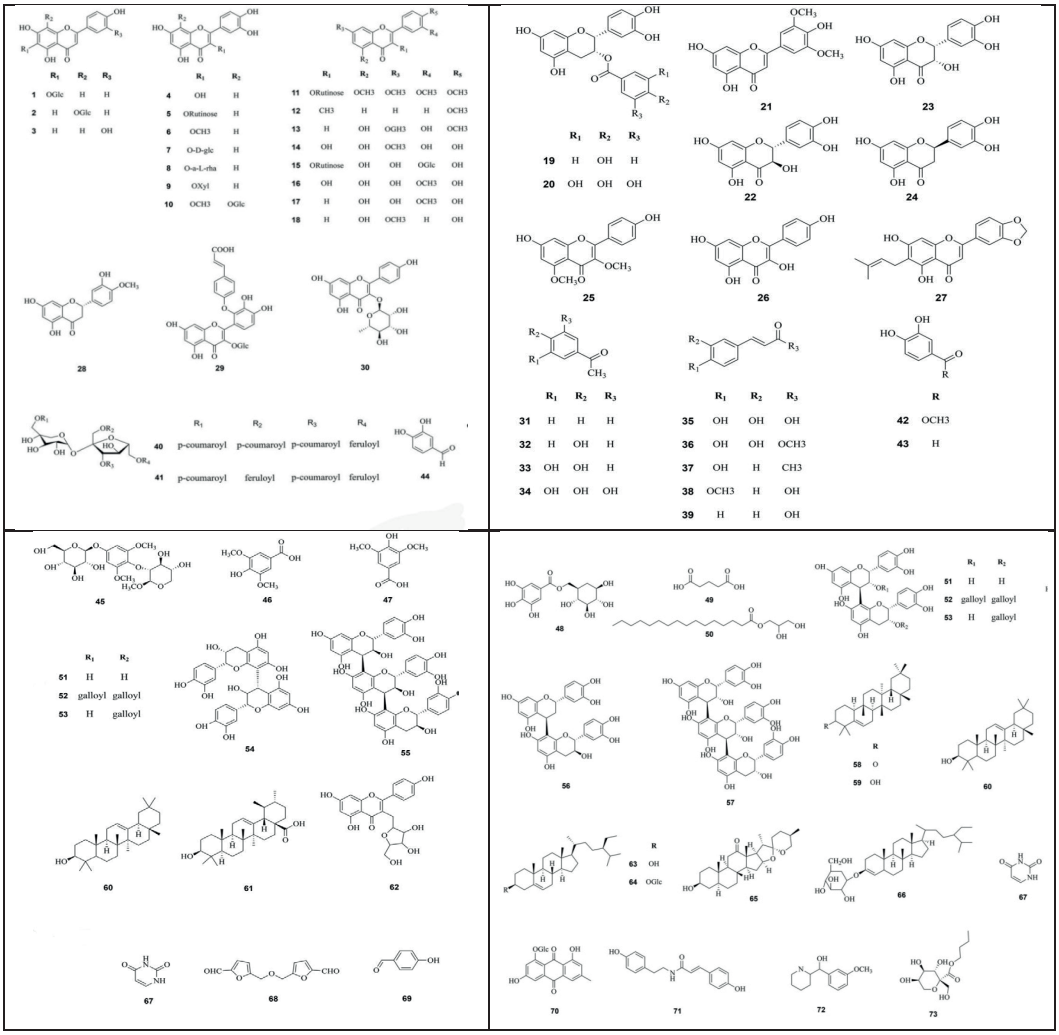


Figure 2. The chemical structures of the isolated components from buckwheat (*Fagopyrum debtors*) (Zhang et al., 2021)

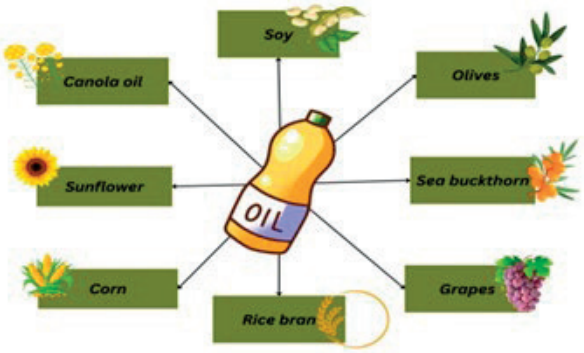


Figure 3. Different oils used for food oleogels (Perța-Crișan et al., 2023)

Table 2. Oleogels used in m eat products (Pușcaș et al., 2020)

The types of Oleogels analyzed and their references	Meat products	
	Structuring Strategy	Conclusions
Oil of gelled sunflower oil with 20% monoglyceride and phytosterol blend (Kouzounis et al., 2017)	Direct dispersion of oleogelators in oil, followed by cooling to room temperature	It was replaced with 50% of the fat from pork shoulder from frankfurter sausages with sunflower oil gelled with monoglycerides: phytosterols
Sunflower oil gelled with 10% or 20% γ-oryzanol and β-sitosterol (Panagiotopoulou et al., 2016)	*Y-oryzanol and p-sitosterol* were added to the oil at an elevated temperature and maintained at 90-120°C for 30 minutes under constant stirring, followed by cooling.	50% oleogel was used, replacing the fat from the pork shoulder in the Frankfurt sausages.
Oleogel gelified with 8% beeswax (Franco et al., 2019)	Direct dispersion of beeswax in oil, followed by cooling to room temperature	25% and 50% pork back fat replaced with oleogel in Frankfurt sausages, without affecting the texture
Canola oil gelled with hydroxypropyl methylcellulose (Oh et al., 2019)	Approaching the structure of foam	The 50% replacement of beef tallow in meatballs was generally acceptable
Gelified olive oil with soy protein concentrate and mineral water, replacing 15%, 25%, 35%, 45%, and 55% of the pork (Utrilla et al., 2014)	Olive oil, soy protein concentrate, and mineral water in a 10:1:8 mixture was emulsified	25% of the pork meat replaced with oleogel in salchichon-type sausages resulted in a new product

The meat business is undergoing transformation because of the experimental findings from the examination of meat products that contain oleogels. These goods may serve as a substitute for technological and nutritional input (Bot et al., 2011).

The quality parameters of the products, which must preserve the traditional recipe in newly developed dishes, have a significant impact on consumer preferences (Grasso et al., 2014).

Meat products' structure and flavour are influenced by fats, which also affect their stability, texture, and binding qualities (Pehlivanoğlu et al., 2018). It is crucial that the new formulation preserves the distinctive qualities of traditional beef products since quality factors affect consumer preferences (De Oliveira et al., 2012).

The potential use of oleogels as a healthier substitute for meat has been examined to create meat products with a better nutritional profile and less saturated fat (Bourne, 2002). Without affecting the finished product's physicochemical and sensory qualities, these lipid structures can either completely or partially replace fat (Martins, 2019). The interaction between the meat proteins and oleogel, however, may cause some changes in texture and consistency, which could result in the creation of a more rigid protein matrix in the finished product (Pintado et al., 2020).

Oleogels made from canola, soybean, and flaxseed oils and structured with 10% ethyl cellulose of different molecular weights provide a pertinent example. Therefore, the use of oleogels based on vegetable oils structured with ethyl cellulose can serve as a viable and healthy alternative to traditional solid fats in the food industry, contributing to the improvement of the nutritional lipid profile of products (Álvarez et al., 2011; Barbut et al., 2019). The viability of this strategy has been confirmed by experiments that found that substituting all the beef fat in the frankfurter recipe produced a product with a texture comparable to the traditional version (Barbut et al., 2016).

Using oleogels made from sunflower oil and structured with phytosterols like γ-oryzanol and β-sitosterol at 10% and 20% concentrations was another tactic. In this instance, the reformulation increased the overall lipid content by using oleogel emulsions in addition to partially be substituting fat. The results of

the investigation revealed that the frankfurters' texture did not significantly alter and that the pH and lipid oxidation levels stayed within ideal bounds. The chewability and hardness of the product were impacted by the softer consistency of the varieties that contained oleogel emulsions. Although some emulsion samples were thought to have a distinct texture from the original version, the products were generally highly received in terms of sensory rating.

The replacement of pork fat in frankfurter sausages was the subject of another investigation (Zetzl et al., 2012). A structured oleogel composed of a 3:1 ratio of monoglycerides and phytosterols was used in these sausages. With this composition, 50% of the pork fat could be substituted without noticeably lowering the product's quality. Compared to oleogels made entirely of monoglycerides, the combination of monoglycerides and phytosterols produced a more robust network with better hardness, stability, and melting temperatures (Ioniță-Mîndrican et al., 2022).

The cohesiveness and elasticity of the reformulated banger recipes were comparable to those of the original recipes; nevertheless, variations were observed in terms of gumminess, brittleness, and hardness. The new goods' acceptability was validated by sensory evaluation, and there was no discernible change in the oxidation levels.

To produce a healthier alternative to typical pork, a sort of salchichon sausage made from deer meat was made using an oleogel based on olive oil that was emulsified with soy protein and water. Several salchichón samples were made, with the control version comprising 25% pork and 75% lean venison. Pork was progressively substituted in the oleogel samples at 15%, 25%, 35%, 45%, and 55% (Beriain et al., 2011). As anticipated, the content of monounsaturated fatty acids increased with the amount of olive oil oleogel (Bloukas et al., 2002).

The analyzed and reformulated samples with oleogel exhibited similar physicochemical properties during baking, including mass loss, pH, water activity, and instrumental color analysis. Additionally, the degree of lipid oxidation and the acidity index remained within acceptable limits, confirming the product's stability (Davidovich-Pinhas, 2016; Mei et al., 2006). All types of salchichon reformulated with oleogel were considered acceptable, but those that replaced up to 25% of the pork meat received the highest scores from consumers (Yu et al., 2012). This suggests that there is an optimal threshold for substituting animal fats, so that the texture and taste remain appealing to consumers. The oleogel-reformulated salchichón contained twice as much oleic acid compared to the traditional recipe (Utrilla, 2014). This aspect highlights the nutritional benefits of using oleogels, contributing to a healthier lipid composition by increasing the content of unsaturated fatty acids. Following the study, the gelling of olive oil with soy protein concentrate and mineral water was used to replace 15%, 25%, 35%, 45%, and 55% of the pork (Utrilla et al., 2014). The formulation with 25% substitution proved to be the most effective, resulting in a new type of salchichon product that was well accepted by consumers. Therefore, the use of oleogels in the reformulation of salchichon allows for the partial replacement of animal fats, maintaining the physicochemical stability and acceptability of the product. The optimal level of pork fat substitution seems to be 25%, ensuring a balance between improving the lipid profile (more oleic acid) and maintaining the texture and flavor desired by consumers. This confirms that oleogels represent a viable and healthy alternative in the food industry for reducing saturated fats in meat products.

CONCLUSIONS

According to the analysis of the specialised literature, adding buckwheat to smoked sausages has a favourable effect since it enhances the nutritious value of the product. The balanced spectrum of key amino acids found in buckwheat proteins can improve the sausages' nutritional value. Furthermore, fibre can aid in improved digestion, and antioxidants such rutin support the preservation of lipids' oxidative stability, hence prolonging the product's shelf life.

In addition to its nutritious value, buckwheat affects the smoked sausages' texture. It can

improve the product's juiciness and tenderness and give it a more palatable consistency because of its capacity to absorb water. Buckwheat's high starch content also helps the ingredients stay cohesive by strengthening the bonds between them and keeping the sausages from breaking.

When animal fat is partially or completely replaced with oleogel—which is made by structuring sunflower, canola, flaxseed, soybean, and olive oils—the amount of stearic and palmitic acids is greatly decreased, the amount of linoleic acid is increased, and the organoleptic profile of fatty acids is improved—all while preserving good organoleptic qualities.

The overall fatty acid profile improved as the amount of monounsaturated fatty acids and polyunsaturated fatty acids rose. The microstructure of the semi-smoked sausages was not significantly altered by the inclusion of oleogels.

In order to produce healthier sausages with better fatty acid profiles and sensory qualities, it is therefore possible to suggest using oleogel in place of pork fat.

As a result, these developments mark a significant advancement in the creation of more accessible and nutritious food items that meet contemporary dietary needs.

The evidence provided leads to the conclusion that goods with additional plant proteins are now a viable substitute and, more importantly, essential for maintaining customers' nutritional balance. These products stand out due to their nutritional qualities.

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THE IMPACT OF THE THERMAL PROCESS APPLIED ON POLYCYCLIC AROMATIC HYDROCARBONS IN WILD BOAR MEAT

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Abstract

Thermal processes applied to meat significantly influence its chemical composition. Thus, meat is considered an essential food due to its high nutritional value, good digestibility and appreciated culinary quality. PAHs in preserved products vary significantly, due to differences in preservation processes, chemical composition of the product, fat and water content. These organic compounds are formed from aromatic nuclei combined during pyrolysis or incomplete combustion of organic matter. The HPLC method was used to analyze polycyclic aromatic hydrocarbons. Following the study conducted on wild boar meat, it was found that all PAHs had the lowest values in the case of the boiling process. The highest average content was represented by the following hydrocarbons: phenanthrene (95.43 µg/kg) followed by naphthalene (46.55 µg/kg), fluoranthene (18.16 µg/kg), pyrene (10.24 µg/kg) and chrysene (9.78 µg/kg), all identified in wild boar meat (in sunflower oil). The aim of the study was to analyze polycyclic aromatic hydrocarbons in wild boar meat under the influence of thermal processes and under the influence of the vegetable (sunflower oil) and animal (lard) sources used for cooking.

Key words*: lard, oil, PAH, thermal processes, wild boar meat.*

INTRODUCTION

The food chain is the main source of contamination with polycyclic aromatic hydrocarbons (PAHs), a statement supported by research in the specialized literature (Duedahl-Olesen & Ionas, 2021).

Game meat is a main source of protein that is increasingly sought after nowadays, in many developed countries including Europe (Vidrean et al., 2024).

An inadequate culinary education leads to the formation of polycyclic aromatic hydrocarbons both in meat from domestic animals and in meat from game animals (Lee et al., 2016). PAHs are considered to be carcinogenic compounds for humanity, with a high degree of triggering of oncological diseases but also of other diseases associated with this disease (Diggs et al., 2011). According to the existing data in the specialized literature, beanzo[a]pyrene, is a compound that is part of category a and b, being considered carcinogenic, capable of producing both genotoxic and mutagenic effects (Ramesh et al., 2004; Mastan et al., 2023). Currently, there are various concepts regarding the thermal processes used to reduce the formation of PAHs in meat (Ciecierska et al., 2023).

The correct selection of oil or lard used, as well the temperature and humidity are essential points to have an acceptable concentration of PAHs (Diggs et al., 2011; Vidrean et al., 2024). Levels of PAHs are detectable in all internal organs according to data from the specialized literature, and especially in organs with rich adipose tissue, these being considered a constant reservoir for the gradual elimination of PAHs (Ciecierska & Obiedzinski, 2007).

PAHs have the following essential characteristics: high molecular mass, at room temperature they become solid, are insoluble in water, can be oxidized and degraded in the presence of light and thus have a lower molecular mass (Duedahl-Olesen & Ionas, 2021; Mastan et al., 2023).

Contaminants such as PAHs represent an important family of toxic substances, which are

also ubiquitous in the environment, and human contamination can also be achieved by inhaling cigarette smoke and the most important source of contamination being the dietary intake of contaminated food due to incorrect thermal processing (Kazerouni et al., 2001; Djinovic et al., 2009; Vidrean et al., 2024). These contaminants can reach the body including through contaminated air due to exhaust gases produced by cars or industry (Ramesh et al., 2004).

PAHs constitute a class of pollutants resulting from the process of incomplete combustion of organic matter (Nielsen et al., 1996; Ferhadian et al., 2010; Savin et al., 2024). Several types of meat that are subjected to thermal cooking processes, especially smoking and grilling, trigger the formation of PAHs (Hitzel et al., 2013; Olatunji et al., 2022). High heat processes through the use of the grill or through the use of coal or wood can lead to an increase in the concentration of PAHs (Barranco et al., 2004; Ifeyinwa et al., 2024).

In the case of the roasting process, the meat may present levels higher than 10-20 pg/kg, Lee et al., 2016, states that these compounds can have many serious effects on the body (genotoxicity, carcinogenicity, immunotoxicity, teratogenicity). The major role of meat processing is to improve its quality, by changing the sensory properties and helping the digestion process, including obtaining a longer shelf life of meat-based preparations (Biesalski, 2005; Wretling et al., 2010). Considering the increasing consumption of meat, it is mandatory to study the degree of meat contamination due to thermal processes and the way in which they are carried out (Halagarda & Wojciak, 2022).

MATERIALS AND METHODS

The samples in the case of this study were represented by meat from the wild boar from the Transylvania region. The aim of this study was to determine the level of PAHs in wild boar meat after applying the thermal process of frying, boiling and grilling. Animal lard and sunflower vegetable oil were used in the thermal frying process. The wild boar meat was cooked as follows: grilled, at a temperature of 190°C, for 50 minutes; fried in oil and lard at a temperature of 180°C, for 40 minutes; boiled at a temperature of 120°C, for 30 minutes.

To determine the level of PAHs, the chromatographic method was applied, using the Perkin-Elmer HPLC system. The degassing system used was: model 200 series; Manufacturer Perkin-Elmer, binary pump, automatic sample introduction system: model Flexar. With the help of the thermostat (model Spark), temperature control is ensured in the range of 10ºC-90ºC. The fluorescence detector (200 series; Manufacturer Perkin-Elmer), has a double monochromator - excitation and emission; the wavelength range is: excitation 200-850 nm; emission 250-900 nm. The chromatographic columns used: ZORBAX Eclipse PAH; Manufacturer Agilent Technologies with column dimensions of 4.6×150 mm, diameter of 5 µm.The principle of the method consists in the use of high-performance liquid chromatography (HPLC), with fluorescence detection after the solid-liquid extraction stage to determine the 15 PAHs in wild boar meat. Interferences are represented by asymmetric peaks or peaks that are wider than those corresponding to the reference compound.

Cases of interference can exist, for example, in the case of naphthalene and phenanthrene. Reagents used are of recognized analytical quality. We used blank samples to guarantee that the reagents do not contain PAHs in detectable concentrations. The working method includes three important stages: the extraction stage, the measurement stage and the stage of expressing the results. The extraction stage includes: the exact weighing of 10 grams of wild boar meat which is homogenized with the help of a blender, then we added 50 ml of 0.4 M KOH solution in ethanol and water (9:1) for saponification, which is carried out in an ultrasound bath for 30 minutes. Filtration is carried out with the help of filter paper, the obtained product was extracted twice with 15 ml of cyclohexane, then the obtained supernatant was purified on a Florisil column, followed by evaporation to dryness under nitrogen flow, and finally it is brought back with 1 ml of acetonitrile. Before the injection process, the samples will be filtered on 0.45 µm cartridges. For the measurement step, a gradient and wavelength program was used to identify the 15 PAHs. To express the results, we used a calculation formula that includes

several elements: the concentration read by HPLC, the dilution factor, the mass of the sample in grams and the % recovery.

RESULTS AND DISCUSSIONS

Meat and meat products are widely consumed, especially smoked and grilled (Ciecierska & Obiedzinski, 2007). The data from the specialized literature are still not sufficient about the acceptable levels of PAH, therefore a more detailed investigation of the meat subjected to different thermal cooking processes from different species of animals is needed, considering the high risk of affecting the human body.
Figures 1-5 show the average values for polycyclic aromatic hydrocarbons analyzed from wild boar meat depending on the treatment applied and the fat source (sunflower oil and pork lard). Other studies were conducted by: Mastan et al. (2023), who evaluated the influence of heat treatment on PAH in fish; the impact of wood used for smoking and fish species on PAH content in smoked fish (Savin et al., 2024); the content of polycyclic aromatic hydrocarbons (PAH) in chicken, turkey and duck meat after exposure to traditional smoking with different types of wood and different periods of smoke exposure (Coroian et al., 2023).
According to the graph above (Figure 1), the highest average value is represented by naphthalene in the game meat sample through the thermal process of frying in sunflower oil.

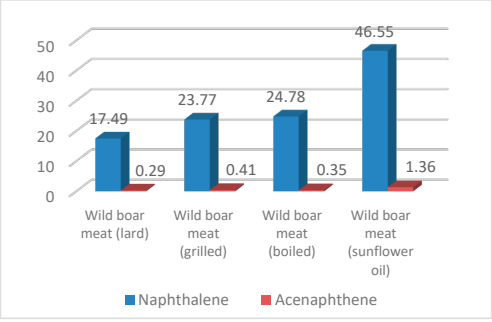


Figure 1. Naphthalene, acenaphthalene from wild boar meat (in lard), wild boar meat (grilled), wild boar meat (boiled), wild boar meat (in sunflower oil) (µg/kg)

Acenaphthene shows lower average values compared to naphthalene in all thermal processes used for game meat (Figure 1). The lowest average values for both indicators are found in the game meat sample subjected to the frying process in lard, and the highest values are in the case of the sample subjected to the sunflower oil frying process (Figure 1).
In the study carried out by Viegas et al. (2012), the authors found that meat subjected to grilling, using charcoal for fire, observed that maintaining stable combustion leads to a decrease in PAHs. One of the most important triggering factors that lead to the production of PAHs through the grilling process, is the smoke that comes from the incomplete burning of the fat that comes into contact with the flame (Viegas et al., 2012).
According to the study carried out by Rose et al. (2014) the authors of the study draw attention to the consumption of food on the grill, because enormous amounts of PAHs can be formed, which are consumed in a single meal. In the case of salmon grilled with coconut shell charcoal, the authors of the study concluded that the levels of PAHs showed a significant decrease (Rose et al., 2014). Grilling with charcoal and wood chips leads to the formation of benzo(a)pyrene in most food samples subjected to the thermal grilling process (Rose et al., 2014). Food that is cooked closer to the heat source leads to an increase in the level of PAHs, and the cooking time can lead to a moderate increase in the level of PAHs (Rose et al., 2014; Onopiuk et al., 2021).
The lowest average value of fluorene can be observed in the case of the sample subjected to the boiling process, with a value of 7.96 µg/kg, compared to phenanthrene, where the lowest average value is in the case of the sample subjected to the process of frying in lard, with an average value of 16.71 µg/kg (Figure 2).

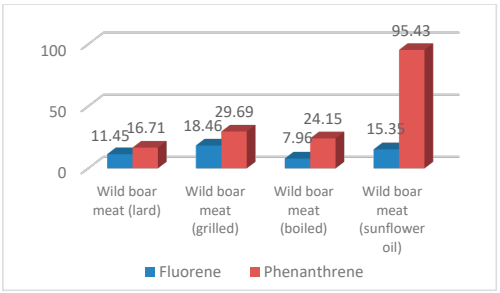


Figure 2. Fluorene, phenanthrene from wild boar meat (in lard), wild boar meat (grilled), wild boar meat (boiled), wild boar meat (in sunflower oil) (µg/kg)

The following factors can contribute to the increase or decrease of PAH depending on the management mode: the heat source, the type of grill used horizontally/vertically, the species from which the meat comes, as well as other parameters, for example the degree of maturation of the meat, marinating or even the addition of spices (Duedahl-Olesen & Ionas, 2021).

The results of the study carried out by Khan et al. (2008), confirm the fact that PAHs are both pyrogenic and petrogenic.

Ifeyinwa et al. (2024), detected the following values regarding the concentrations of PAHs: total PAHs (mg/kg) in the samples of game meat subjected to the smoking process obtained the following values 2.763 ± 0.185 mg/kg, in the mackerel meat subjected to the smoking process they obtained 1.618 ± 0.102 mg/kg and in the catfish meat subjected to the process of smoking obtained 1.718 ± 0.129 mg/kg, while the values in chicken meat subjected to the smoking process were 0.771 ± 0.049 mg/kg.

Maximum average of 222.7 ± 13.2 µg/kg, of the 16 PAHs studied, was detected in the fish samples subjected to the smoking process, and a minimum average of 112.9 ± 7.2 µg/kg, in the case of chicken kebab, for the same studied PAH (Ifeyinwa et al., 2024).

Anthracene and fluoranthene show the highest values according to Figure 3, in the case of the sample subjected to the frying process in sunflower oil. The average values for these two indicators are low for the rest of the processes, namely: fried in lard, grilled and boiled (Figure 3).

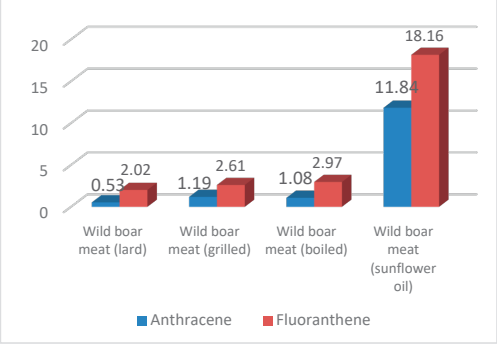


Figure 3. Anthracene, fluoranthene from wild boar meat (in lard), wild boar meat (grilled), wild boar meat (boiled), wild boar meat (in sunflower oil) (µg/kg)

The results of a study carried out in the city of Togo on samples of smoked fish, indicate concentrations above the permissible limit for benzo[a]pyrene (BaP), benz[a]anthracene, (BaA), benzo[b]fluoranthene (BbF) and chrysene (CHR); The daily dose can range from 23.22 to 130.09 ng/kg/day for the four PAHs and from 1.4 to 31.64 ng/kg/day for BaP. (Bouka et al., 2020).

An experiment carried out in Estonia, to determine the level of PAHs, from meat samples from the trade respectively grilled meat samples, after the study the results indicated values that exceeded in 3.4% of cases the limit allowed for the benzo[a]pyrene indicator (Reinik et al., 2007).

A study carried out on samples of smoked sausages, pork bacon, ham, grilled pork and poultry from Cyprus, the authors observed the highest concentrations of PAHs in the samples that were kept in the thermal process for a longer time and that have a higher fat content, also in this study it was specified that 12% of the products subjected to the smoking process and 15% of the products subjected to the thermal process at grill have exceeded the limits allowed by the legislation of the European Union (Kafouris et al., 2020).

The highest average value in the case of the benzo[a]anthracene indicator is in the meat sample subjected to the frying process in sunflower oil, with a value of 3.29 µg/kg, and the lowest average values are in the case of the boiling and frying lard (Figure 4).

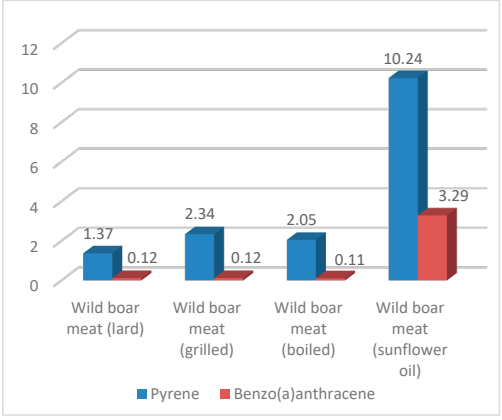


Figure 4. Pyrene, benzo[a]anthracene from wild boar meat (in lard), wild boar meat (grilled), wild boar meat (boiled), wild boar meat (in sunflower oil), (µg/kg)

Choosing an appropriate type of thermal process, respectively the raw material with the lowest fat content, can be an effective option to reduce the amount of PAHs inside meat preparations (Ciecierska et al., 2023).

We find the highest average values for chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo [ghi]perylene and indeno[1,2,3-cd], in the case of the process of frying the venison sample in sunflower oil, according to Figure 5. The undetectable values were in the case of the following indicators: benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene and benzo [ghi]perylene from the samples subjected to the processes frying in lard, boiled, grilled and frying in sunflower oil (Figure 5).

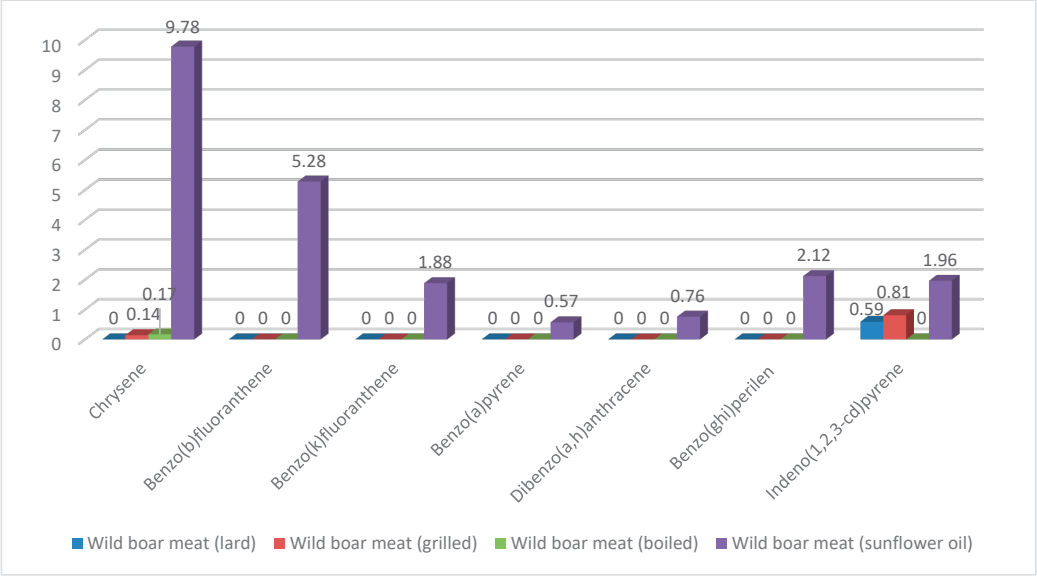


Figure 5. Chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[ghi]perylene and indeno[1,2,3-cd], pyrene from wild boar meat (in lard), wild boar meat (grilled), wild boar meat (boiled), wild boar meat (in sunflower oil) (µg/kg)

CONCLUSIONS

Game meat, in this case wild boar, can contain different concentrations of PAHs, with the highest average values in the frying process in sunflower oil, according to our study. The highest average value for benzo[a]anthracene is in the case of frying in sunflower oil. It is essential to remember that a regular consumption of game meat subjected to various thermal treatments, which give the average values of PAHs above the limits allowed by the legislation, over a prolonged period of time, may present a possible carcinogenic risk for consumers. The method and technique chosen for preparing the meat is important. The consumer is concerned about the special sensory value that is obtained as a result of the various thermal processes of meat preparation, but these compounds that result from the thermal processes and which are obviously not friendly to the human body must be taken into account.

Among the PAHs studied by us, the highest values are for acenaphthalene with an average value of 46.55 (µg/kg), phenanthrene with an average value of 95.43 (µg/kg), fluoranthene with an average value of 18.16 (µg/kg), pyrene with an average value of 10.24 (µg/kg) and chrysene with an average value of 9.78 (µg/kg), all these indicators being with higher average values in the case of game meat subjected to the frying process in sunflower oil compared to the other three processes used, boiling, frying in lard and grilling, where the average values were lower.

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ARONIA ENHANCED CACIOTTA AS A DAIRY ALTERNATIVE WITH IMPROVED FUNCTIONAL PROPERTIES

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Abstract

The study aimed to assess the impact of incorporating 5% aronia powder on the physicochemical and functional properties of two varieties of caciotta: one with homogenized aronia powder and another with aronia powder layered throughout. The results indicated an overall decrease in fat and crude protein content for both aronia variations compared to the control, while carbohydrate levels exhibited a slight increase. A substantial enhancement in antioxidant activity was seen in both varieties of enhanced caciotta (40.61 ± 0.32 µMol Trolox/g dw for the homogenized version and 39.3 ± 0.30 µMol Trolox/g dw for the layered variety) in comparison to the control (2.7 ± 0.52 µMol Trolox/g dw). Anthocyanins varied between 0.91 ± 0.014 mg C3G/g dw (homogenized) and 0.82 ± 0.01 mg C3G/g dw (layered), whereas polyphenols increased from 1.1 ± 0.01 mg GAE/g dw to 3.4 ± 0.01 mg GAE/g dw. Consequently, the integration of aronia powder into caciotta is an effective approach for creating a functional product with antioxidant properties and beneficial health effects, aiding in the mitigation of oxidative stress and the prevention of chronic diseases.

Key words: *antioxidant activity, dairy products, functional yogurt, plum pomace, pigments.*

INTRODUCTION

Currently, there is an increasing public interest in health-promoting foods, particularly health-promoting dairy products, which have substantial popularity among consumers.

Cheese is typically a nutrient-rich, and easily digestible fermented milk product appreciated globally, the beneficial effects of its consumption being continuously discussed. Cheese is a major source of quality proteins, fats, vitamins, minerals, probiotics, and bioactive compounds, which can provide considerable health benefits (Zhang et al., 2023). Conversely, cheese possesses elevated levels of saturated fats and sodium, which are regarded as detrimental dietary factors for cardiovascular health (Givens, 2022).

The term Caciotta originates from the Italian word "cacio", a colloquial designation for cheese (Mucchetti et al., 2006). The organoleptic characteristics of this cheese may differ based on the traditions of the many regions of the country in which it is manufactured (Gobbetti et al., 2018). Caciotta is typically classified as a semi-soft cheese with a short to medium maturation period, weighing approximately 1 kg, and is made from either pasteurized whole cow's milk exclusively or a blend of cow's and ewe's milk. The taste of caciotta might differ based on the production region, maturation period, and type of milk chosen. The aroma profiles of cow, ewe, and goat milk are distinct, with cow milk recognized as having the smallest variety and number of aroma components (Bancalari et al., 2020).

The production of dairy products enhanced with natural additives offers a natural appealing to these types of products (Granato et al., 2018). These supplements, in addition to being widely acknowledged as safe, enhance the flavour and colour of dairy products (Andersen et al., 2023). Vegetable by-products have confirmed significant amounts of carotenoids, fibers,

polyphenols, vitamins, tocopherols, as well as other nutrients. The by-products have environmental costs and, more importantly, possess unexploited ability to provide nutritional enhancement (Lipșa et al., 2023).

The functional properties of these compounds can improve foods and satisfy customer demands for products with fewer artificial ingredients, higher authenticity and increased levels of nutrients (Abou-Zeid, 2016). In the present study, we enhanced a caciotta cheese model by incorporating aronia powder. The choice of this substrate was supported by research regarding its several potential bioactive benefits for human health, including antidiabetic, antiobesity, and antioxidant capabilities, as well as its impact on cardiac, hepatic, and neuroprotective functions (Ren et al., 2022).

As mentioned by Ren et al. (2022), phenolic substances, including anthocyanins, cyanidins, phenolic acids, proanthocyanidins, triterpenoids, and their derivatives, have been recognized as the primary active constituents of Aronia berries. The study aimed to evaluate the impact of incorporating 5% aronia powder on the characteristics of caciotta cheese by comparing two distinct technological methods of incorporation (homogenization and stratification) regarding the physicochemical, phytochemical, color characteristics, and textural quality of the final product, in comparison to a control sample.

MATERIALS AND METHODS

Raw milk received from the Rediu, Iași Research Station of the University of Life Sciences underwent specific physicochemical analyses (pH, moisture, fat, protein, total solids, solid non-fat) employing the Association of Official Analytical Chemists (AOAC) procedures (Rațu et al., 2021).

This study employed some of the following reagents and chemicals: Folin-Ciocalteu reagent, acetone, methanol, hexane, ethanol, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium acetate solution, gallic acid, sodium carbonate and aluminum chloride. All the chemicals were purchased from Sigma Aldrich, Steinheim (Darmstadt, Germany).

Origin and preparation of aronia pomace powder (AP)

The aronia berries were harvested at the end of August 2024, with the color and flavor of the fruits serving as indicators of ripening. They were effortlessly separated from the clusters, exhibited an intense black-violet hue, and a mildly astringent flavor with a sweet-sour undertone. The fruits were manually harvested, and the conditioning procedure was conducted at a temperature of 2-4°C until they were processed. The aronia juice was extracted using a Bosch MES3500 juicer (Philips Consumer Lifestyle B.V., Drachten, Netherlands) shortly after the berries were washed with distilled water to remove impurities. The resulting pomace was retained for further processing. The particles were then ground in a laboratory mill for 60 seconds to achieve a size of 450 µm, and the drying process was conducted using heated air convection at a temperature of 45°C for 15 hours through a food dehydrator. In addition, the powder was sterilized with a UV lamp to prevent potential microbiological contamination.

Extraction of biologically active compounds from AP

Biologically active substances were extracted from aronia powder (AP) via ultrasound-assisted extraction. Specifically, 1.0 g of AP was mixed with 10 mL of a 70% ethanol solution, acidified with citric acid in an 8:2 (v/v) ratio, and then underwent an ultrasound treatment for 30 minutes at 42°C and 40 kHz using an Elmasonic S 180 H sonication water bath (Elma, Germany). The obtained supernatant was collected and centrifuged for 5 minutes at 6000 rpm and 4°C. The AP extract was subsequently used to assess the antioxidant activity of phytochemicals, including anthocyanins, polyphenols, and flavonoids.

Antioxidant activity (DPPH)

The extract's antioxidant activity was evaluated using the DPPH radical technique, as described by Lipsa et al. (2024). This mixture was obtained by combining 100 µL of extract and 3.9 mL of a 0.1 M DPPH reagent. The solution was kept at 25°C for 30 minutes in dark conditions. The absorbance of the mixture was measured at a wavelength of 515 nm utilizing a UV–vis spectrophotometer (Analytik Jena-Specord 210

Plus, Germany). The blank solution was obtained by mixing 3.9 mL of 0.1 M DPPH reagent with 100 µL of methanol, and the absorbance of the mixture was evaluated accordingly. The results were reported as µmol Trolox equivalents (TE)/g dry weight, derived from a curve of calibration. The radical scavenging activity was subsequently quantified as a percentage of inhibition utilizing the following formula:

$$I(\%) = \frac{A_b - A_s}{A_b} \times 100,$$

where A_b is the absorbance of the blank solution and A_s is the absorbance of the analyzed sample.

Total monomeric anthocyanin content

The total monomeric anthocyanin concentration was determined using a modified pH differential method, as outlined by Lipsa et al. (2024). Prior to analysis, the samples were diluted at a ratio of 1:10. The absorbance of the diluted extracts was measured at two specific wavelengths, respectively 520 nm and 700 nm, utilizing a UV-Vis spectrophotometer (Analytik Jena - Specord 210 Plus, Jena, Germany). For each measurement, 200 µL of extract and 800 µL of buffer solution at pH 1 and pH 4.5 were employed. The results were quantified in milligrams of cyanidin-3-glucoside (C3G) per gramme of dry weight (dw).

Total polyphenolic content

The total polyphenol content of AP extract was quantified using the Folin-Ciocalteu technique, as outlined by Gavril et al. (2024), alongside spectrophotometric analysis. Consequently, 200 µL of the extract was combined with 15.8 mL of distilled water and 1 mL of Folin-Ciocalteu reagent. After 10 minutes, 3 mL of a 20% sodium carbonate (Na_2CO_3) solution was added. The acquired solution was maintained at room temperature in dark conditions for 60 minutes. The absorbance was afterwards measured at a wavelength of 765 nm using a UV-Vis spectrophotometer (Analytik Jena Specord 210 Plus, Germany). The results were quantified in milligrams of gallic acid equivalents per gramme of dry matter (mg GAE/g dw).

Total flavonoid content

The total flavonoid content of the chokeberry powder extract was quantified by applying the aluminum chloride spectrophotometric technique as outlined by Lipsa et al. (2024). The procedure involved the preparation of a mixture by combining 0.25 mL of extract with 2 mL of distilled water, thereafter, adding 0.075 mL of a 5% sodium nitrite ($NaNO_2$) solution. After 5 minutes, 0.15 mL of aluminum chloride ($AlCl_3$) solution was added, followed by the addition of 0.5 mL of 1 M sodium hydroxide (NaOH) solution after another 6 minutes. The absorbance of the analyzed solution has been measured at a wavelength of 510 nm, utilizing a UV-Vis spectrophotometer (Analytik Jena Specord 210 Plus, Germany). The total flavonoid content was quantified in milligrams of catechin equivalents per gramme of dry weight (mg CE/g dw).

Preparation of caciotta enhanced with AP

Caciotta cheese was produced by adapting a technological workflow as described in Figure 1. The process started with the quantitative and qualitative reception of fresh milk, followed by filtration and pasteurization at 60°C for 30 minutes. The milk was chilled to 36°C after pasteurization and subsequently inoculated with an aromatic mesophilic starter culture of the LD type (*Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris*).

The microbial rennet (CHY-Max M Liquid) was introduced after inoculation and homogenization, as determined by the specific quantity. Coagulation took place at 36°C for 35-40 minutes. To facilitate the dehydration of the curd, the curd was initially cut into small pieces and subsequently partially removed approximately 30% of the whey. Subsequently, the curd was slowly heated to 42°C while being continuously stirred for 10-15 minutes. After this phase, an additional 30% of the whey was removed. At this point, 3 batches of caciotta cheese were formed (caciotta without AP - *CWAP*, homogenized AP caciotta - *CHAP* and layered AP caciotta - *CLAP*).

The inclusion of aronia powder (5%) was accomplished through either homogenization or stratification, dependent upon the experimental variant. For CHAP variant, the powder was subsequently incorporated directly into the forms during the curd pouring process.

For the CLAP, the curd was poured into cylindrical forms in two phases for the caciotta with aronia powder in layers, with the

introduction of aronia powder between the two layers. All variants were subjected to a pressure of 3-6 kg/kg cheese and rotated 2 times during the 14-hour pressing process. Immersion in a 12% saline solution for 36 hours was the method of salting (Figure 1).

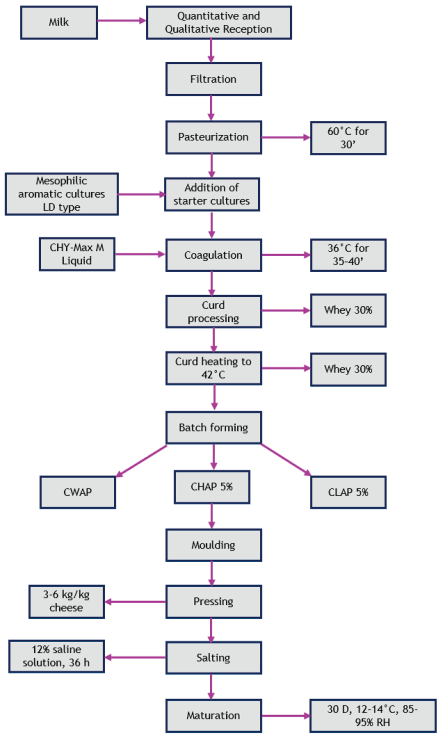


Figure 1. Flow diagram of aronia enhanced caciotta

After the salting process, the cheeses were dried at ambient temperature and subsequently matured for 30 days in spaces with a relative humidity of 85-95% and temperatures of 12-14°C. The cheeses were rotated every 4-6 days during the maturation process. For specific mold development, the cheeses were rinsed with brine three times per week.

The methods suggested by the AOAC were utilized to evaluate the samples' pH, fat, ash, moisture content, and total protein (Usturoi et al., 2017).

Colour analysis

The colour of the aronia powder and control caciotta was evaluated using the portable colorimeter C-MINOLTA Chroma Meter CR-410 (Konica Minolta, Osaka, Japan). The color parameters determined were L*, a*, and b*. Each sample was represented by three different replicates.

Statistical Analysis

The data were statistically analyzed with Minitab 19 and the data analysis toolbox in Microsoft Excel. Experiments were conducted in triplicate, and standard deviations were employed to compute mean values.

RESULTS AND DISCUSSIONS

The measured values for the phytochemical and colorimetric composition of aronia powder indicate a profile abundant in bioactive components with antioxidant properties (Table 1). The total concentration of monomeric anthocyanins was 1.08±0.02 mg C3G/g d.w., indicating a significant presence of anthocyanin pigments. The overall flavonoid concentration (9.85±0.3 mg CE/g d.w.) underscores the significant role of these secondary phenolic compounds in enhancing product stability and providing protection against oxidative stress.

The total polyphenol concentration (20.90±0.1 mg GAE/g d.w.) indicates the functional properties of aronia powder, underscoring its potential as a nutritionally beneficial addition in food products.

Table 1. Phytochemical and colorimetric features of the AP powder

Parameters	AP Powder
Total anthocyanin content (mg C3G/g d.w.)	1.08±0.02
Total flavonoid content (mg CE/g d.w.)	9.85±0.3
Total polyphenol content (mg GAE/g d.w.)	20.90±0.1
Antioxidant activity (DPPH, µmol TE/g d.w.)	23.65±0.10
Inhibition, %	87.55±0.03
L*	24.1±0.18
a*	12.65±0.05
b*	3.44±0.03

The antioxidant activity assessed via the DPPH technique (23.65 ± 0.10 µmol TE/g d.w.) and the notable inhibition percentage (87.55 ± 0.03%) indicate a significant capacity for free radical neutralization, implying a high biological value of aronia powder. The colour indices L*

(brightness – 24.1), a* (red – 12.65), and b* (yellow – 3.44) denote a deeply dark hue, characterized by a predominance of red-purple, typical of aronia fruits abundant in anthocyanins. The disparity between the elevated values of a* and the minimal values of b* underscores the predominance of cold hues, thereby elucidating the visual influence of aronia powder on the food matrix in which it is incorporated, as well as its efficacy as a natural colouring agent.

Table 2. Values of quality parameters in raw milk

Parameters	Mean
Water (%)	87.20±0.06
Fat (%)	3.80±0.08
Protein (%)	3.30±0.04
Total Solids (%)	12.80±0.08
Solid-non fat (%)	9.00±0.07
pH	6.60±0.02

The primary chemical quality indicators for raw milk have been established to assess its quality attributes. Table 2 displays the chemical composition values of bovine milk samples. The raw milk had an average pH of 6.60±0.02, a water content of 87.20±0.06%, and a total solids content of 12.80±0.08%. The mean solid-non-fat content was 9.00±0.07%, while the mean fat content was 3.80±0.08%. The mean protein concentration was 3.30±0.04%. The findings demonstrate that every raw milk quality parameter satisfies the standards for evaluating the milk's overall quality.

The phytochemical profile and DPPH free radical scavenging capacity obtained from both caciotta varieties (homogenized and layered) and simple caciotta reveal that the incorporation of aronia powder positively influences the phytochemical composition of caciotta cheese (Table 3).

Table 3. The phytochemical composition and antioxidant activity of aronia - enhanced caciotta

Parameters	**Type of caciotta**		
	CWAP	**CHAP**	**CLAP**
Total anthocyanin content (mg C3G /100 g d.w.)	-	0.91±0.014	0.82±0.01
Total flavonoid content (mg CE/g d.w.)	0.55±0.05	2.10±0.007	2±0.01
Total polyphenol content (mg GAE/g d.w.)	1.1±0.01	3.4±0.01	3.17±0.02
Antioxidant activity (µmol TE/g d.w.)	2.7±0.52	40.61±0.32	39.3±0.30

The control sample (CWAP), without of aronia powder, displayed a diminished phytochemical profile, characterized by a low concentration of flavonoids (0.55±0.05 mg CE/g d.w.) and total polyphenols (1.1±0.01 mg GAE/g d.w.), with anthocyanins being absent. Concerning the caciotta versions enhanced with aronia powder (CHAP and CLAP), a significant enhancement of these parameters was noted (Table 3). A monomeric anthocyanin level of 0.91±0.014 mg C3G/100 g d.w. was observed in CHAP cheese, which is slightly higher than the 0.82±0.01 mg C3G/100 g d.w. found in CLAP. The findings indicate that the uniform integration of the powder facilitates a more effective distribution of bioactive components within the cheese matrix.

An approximate fourfold increase in total flavonoid content was noted in the two variants with the incorporation of aronia powder compared to the control batch: 2.10±0.007 mg CE/g d.w. (CHAP) and 2.00±0.01 mg CE/g d.w. (CLAP), thereby underscoring the powder's potential as a valuable functional source of phenolic compounds. A similar pattern was observed with total polyphenols, with CHAP cheese exhibiting a value of 3.4±0.01 mg GAE/g d.w., in contrast to CLAP, which recorded 3.17±0.02 mg GAE/g d.w., and the control batch, which showed just 1.1±0.01 mg GAE/g d.w. The values obtained for total polyphenols support the idea that the approach employed in the integration process impacts the fortification level of the final product.

The antioxidant activity levels for the CHAP variations (40.61±0.32 µmol TE/g d.w.) and CLAP (39.3±0.30 µmol TE/g d.w.) were almost 15 times more than that of the control cheese (2.7±0.52 µmol TE/g d.w.). The substantial enhancement in antioxidant activity underscores the efficacy of incorporating aronia powder in improving free radical scavenging capability, with a marginally elevated value for the homogenized aronia variety.

The evaluation of the chemical composition of the three caciotta cheese variants reveals

significant disparities between the control batch (CWAP) and the two enhanced variants containing 5% aronia powder (CHAP and CLAP), particularly regarding moisture, fiber content, carbohydrates, and dry matter (Table 4).

Table 4 Chemical composition of aronia - enhanced caciotta compared to control batch

Parameters	Type of caciotta		
	CWAP	CHAP	CLAP
Moisture (%)	43.70±0.041	44.01±0.007	43.66±0.009
Total solid (%)	12.68±0.031	55.99±0.007	56.34±0.009
Fat (%)	27.99±0.027	25.67±0.01	25.97±0.01
F/NFS (%)	49.71±0.32	45.84±0.006	46.09±0.008
Protein (%)	23.55±0.019	21.80±0.01	21.61±0.01
Ash (%)	3.69±0.024	3.56±0.009	3.79±0.02
Carbohydrates (%)	1.07±0.001	1.57±0.01	1.60±0.04
Fiber (%)	-	3.39±0.009	3.37±0.02

The moisture content for all three analyzed types was comparable, ranging from 43.66% to 44.01%, indicating a consistent level of hydration characteristic for semi-soft cheeses. The dry matter (total solids) was 55.99% for CHAP and 56.34% for CLAP, slightly higher than the control sample's 56.30%.

The results reinforced the stability of the basic formula, demonstrating that the incorporation of aronia powder did not significantly affect the moisture/dry matter ratio of the final product.

A minor decrease in fat content was noted in the samples with the addition of aronia powder (CHAP – 25.67%, CLAP – 25.97%) compared to the control batch (27.99%), which can be attributed to the diluting effect of including a fiber-rich vegetable element, hence increasing the amount of dry matter.

The fat to dry matter ratio (F/NFS) was reduced in CHAP (45.84%) and CLAP (46.09%) samples relative to the control batch (49.71%), indicating that cheeses incorporating aronia powder may possess a more balanced lipid profile and could be healthier than those lacking this addition (Table 4).

The control sample had a higher protein content (23.55%) than the CHAP (21.80%) and CLAP (21.61%) variants. The reduction in protein level in the caciotta batches with aronia powder can be attributed to the diluting effect of the vegetable addition, without adversely affecting the nutritional quality of the enhanced types of cheese.

The ash content for all three types was comparable, ranging from 3.56% to 3.79%, signifying a consistent mineral composition. The somewhat elevated value for CLAP can be explained by the localized concentration of the powder in specific layers.

In terms of carbohydrates, the cheeses supplemented with aronia powder exhibited marginally elevated values (1.57% – CHAP and 1.60% – CLAP) relative to the control batch (1.07%), attributable to the naturally occurring sugars in the aronia powder.

The most significant contribution is from dietary fiber, which is lacking in the control sample but is present in both variants with added powder, in comparable amounts (3.39% for CHAP and 3.37% for CLAP) (Table 4).

Table 5 outlines the results for the colour parameters and the impact of incorporating chokeberry powder on the visual characteristics of the two varieties of Caciotta containing chokeberry powder. The CIELAB colorimetric system (L*, a*, b*) was employed to objectively assess the colour modifications resulting from the enhancement of the examined food matrix with functional ingredients.

Table 5. Colors of CWAP, CHAP, and CLAP types

Caciotta type	Parameters		
	L	a*	b*
CWAP	85.51±0.15	-3.23±0.62	28.89±0.20
CHAP	45.74±0.026	14.97±0.057	3.25±0.039
CLAP	69.33±0.080	8.11±0.10	1.96±0.070

Consequently, the L* parameter (brightness) exhibits significant variation among the examined samples. The control sample (CWAP) exhibited a value of 85.51 ± 0.15, signifying a very light colour characteristic of dairy products without of vegetable additions. For the remaining two types of caciotta, the L* value exhibited a notable decline, particularly in the homogenized sample (CHAP – 45.74 ± 0.026), indicating a clear darkening of the colour.

The value for the layered caciotta (CLAP – 69.33 ± 0.080) was deemed intermediate, indicating a localized distribution of the pigment, attributable to the sample's layered structure. The a* parameter, indicating a propensity towards red (positive values) or green (negative values), was negative for the control sample (–3.23 ± 0.62), signifying a marginally greenish background characteristic of white cheeses. Positive values were observed for CHAP (14.97 ± 0.057) and CLAP (8.11 ± 0.10), indicating a particular inclination towards red, attributable to the presence of anthocyanins in the aronia powder. The observed difference was deemed conclusive evidence of the efficacy of integrating natural pigments, exhibiting a higher intensity in the homogenized caciotta type. The b* parameter (yellow vs. blue) exhibited elevated values in the control sample (28.89 ± 0.20), indicating the subtle yellow tint of the cheese, which arises from its natural fat content and lactic pigments, without any additional ingredients.

The samples incorporating aronia powder exhibited a significant reduction in b* values (CHAP – 3.25 ± 0.039; CLAP – 1.96 ± 0.070), signifying a decrease in the yellow component and a prevalence of cool hues (red-violet), characteristic of pigmentation associated with anthocyanins.

Table 6 illustrates the assessment of the textural profile and the modifications seen in the mechanical properties of caciotta cheese after the incorporation of aronia powder. The results indicated a distinct trend of enhanced hardness and uniformity in the CHAP and CLAP samples compared to CWAP.

Table 6 Texture of enhanced caciotta compared to control batch

Parameter	CWAP Mean ± SD	CHAP Mean ± SD	CLAP Mean ± SD
Hardness, N	4.70 ± 0.21	5.90 ± 0.26	6.50 ± 0.30
Adhesiveness, mJ	0.40 ± 0.09	0.55 ± 0.11	0.53 ± 0.09
Cohesiveness, -	0.37 ± 0.06	0.50 ± 0.06	0.47 ± 0.05
Springiness, -	1.60 ± 0.13	1.85 ± 0.16	1.95 ± 0.16
Gumminess, N	0.75 ± 0.07	0.87 ± 0.09	0.95 ± 0.10
Chewiness, N	0.60 ± 0.06	0.68 ± 0.07	0.73 ± 0.07

The hardness was 4.70 ± 0.21 N for the control sample and increased up to 5.90 ± 0.26 N for the homogenized sample with addition of powder (CHAP). The CLAP sample had the highest hardness value, with an average measurement of 6.50 ± 0.30 N. The observable increase indicates that the aronia powder facilitated the consolidation of the protein network in the samples containing the powder, with a more pronounced effect in the layered sample, where the powder can form denser and more compact sections. The adhesiveness exhibited marginally elevated values in the caciotta with powder addition (0.55 ± 0.11 mJ – CHAP and 0.53 ± 0.09 mJ – CLAP) relative to the control batch (0.40 ± 0.09 mJ), suggesting an inclination towards enhanced resistance to separation, a phenomenon potentially influenced by the presence of vegetable fibers that retain a higher percentage of water within the cheese's microstructure.

The product's resistance to deformation (cohesiveness) exhibited markedly elevated values in samples containing aronia powder (0.50 ± 0.06 – CHAP and 0.47 ± 0.05 – CLAP) in comparison to the control batch (0.37 ± 0.06), underscoring an enhanced structural integrity of the final product. The newer formulations exhibited higher springiness values (1.85 ± 0.16 – CHAP and 1.95 ± 0.16 – CLAP) in comparison to the control batch (1.60 ± 0.13), signifying an enhanced ability to recover post-deformation, which is a favorable sensory characteristic that enhances the perception of freshness. Gumminess and chewiness exhibited a comparable increase. Gumminess: CWAP: 0.75

± 0.07 N→ CHAP: 0.87 ± 0.09 N→ CLAP: 0.95 ± 0.10 N. Chewiness: CWAP: 0.60 ± 0.06 N → CHAP: 0.68 ± 0.07 N→CLAP: 0.73 ± 0.07 N. These findings indicate that the incorporation of aronia powder improves the mechanical structure of the caciotta and enhances its consistency and chew resistance. The CLAP version often had the highest values for most textural characteristics, suggesting that the cheese's layering promotes the development of more compact and resistant sections in contrast to the homogenized varaint (CHAP), which provides a balance between hardness and homogeneity.

CONCLUSIONS

The findings demonstrated the beneficial effect of including 5% aronia powder on the physicochemical, textural, colorimetric, and antioxidant properties of caciotta cheese.

In comparison to the control batch, the caciotta variants enriched with aronia (both homogenized and layered) exhibited an enhanced phytochemical composition, characterized by elevated levels of polyphenols, flavonoids, and anthocyanins, as well as superior antioxidant activity. From a nutritional perspective, the incorporation of aronia powder markedly enhanced the dietary fiber content and diminished the fat level in relation to the dry matter, indicating an increased functional potential of the final product. The colorimetric changes revealed an enhancement of the red-violet hues in the experimental batches, with a more significant visual effect observed in the homogenized variant. Textural assessment demonstrated enhancements in firmness, elasticity, cohesiveness, and chewiness, hence demonstrating the hypothesis that aronia powder favorably impacts the overall protein structure of caciotta.

Consequently, including aronia powder in Caciotta cheese manufacturing may serve as an effective approach to enhance the nutritional and functional attributes of the final product, while preserving its physicochemical and sensory properties. The findings of this study encourage the incorporation of this natural ingredient into the formulation of novel dairy products that may offer health benefits to consumers.

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REDUCING FOOD WASTE: STRATEGIES, IMPLICATIONS AND FUTURE DIRECTIONS

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Abstract

Food waste is a critical global issue with environmental, economic, and social consequences. This article explores the causes of food waste across the supply chain, from production and processing to distribution and consumption, highlighting its regional and global impacts. Using data from sources like the United Nations Environment Programme (UNEP), Eurostat, and national studies, it provides statistical insights into regional disparities and the effects of policies, economic factors, and social behaviors. The analysis focuses on the European Union and Romania, offering comparative perspectives. To combat food waste, the article proposes strategies, including technological innovations, to enhance supply chain efficiency and minimize losses. Policy interventions at local and national levels are emphasized to establish sustainable practices. Consumer education is identified as essential for raising awareness and fostering better habits. By addressing these key areas - technology, policy, and education - the study advocates for sustainable food systems to reduce waste and its harmful effects on the environment and society.

Key words*: consumption, distribution, food waste, strategies, supply chain.*

INTRODUCTION

Food waste, which refers to the disposal of edible food throughout various stages of the supply chain, is a pressing global issue. Shockingly, about one-third of all food produced for human consumption is either lost or wasted each year, amounting to roughly 1.3 billion tonnes (Gustavsson et al., 2011). This waste not only plays a significant role in contributing to nearly 10% of global greenhouse gas emissions but also leads to considerable economic losses (UNEP, 2024). In the European Union (EU), food waste is estimated at 59 million tons annually, which translates to about 132 kilograms per person (Eurostat, 2024). Romania, as a member of the EU, encounters its own set of challenges related to food waste, significantly affecting both its economy and food security. This article seeks to explore the causes and ramifications of food waste in different regions while proposing effective strategies for its reduction.

Food waste and food loss are similar by the fact that they both occur at each stage of the supply chain, including primary production, processing, at retailers, within the food service industry, and in households.

It is crucial to understand where food is wasted and how much is wasted along the supply chain to devise interventions to reduce wastage and to quantify baselines and progress towards Sustainable Development Goal (SDG).

It is important to consider the definitions of Food Loss and Food Waste and where they arise in the food supply chain (Murphy et al., 2024)

Food waste: typically refers to food that was not ultimately consumed by humans and that is discarded; it can arise intentionally or unintentionally from the human food supply chain in retail, restaurant, food service and household (Qi & Roe, 2019) settings.

Food loss: refers to a decrease in the mass or quality of food before it reaches the consumer; includes all quantities of crop, livestock and human-edible commodities which, discarded or otherwise, do not re-enter the food supply chain in any other utilisation in other contexts such as

in animal feed or for industrial purposes (Santeramo et al., 2021).

Food loss may be due to: harvest, storage, processing, distribution and transportation. Harvest losses refer to the loss of food that occurs during harvesting, often attributed to improper harvesting techniques or timing: early harvesting with a higher moisture content or insufficient drying processing increase the risk of mould growth. It is also referring to food that was not harvested due to labour shortages or low market prices and remained on the field; or to food which not conform to aspect requirements set by retailers: e.g. it is estimated that 25-30% of carrots do not reach the market due to failure to achieve aspect standards (Alvarez de los Mozos et al., 2020).

Storage losses involve spoilage of during storage, either in warehouses or refrigerated facilities. Factors such as unstable moisture control, contamination and temperature fluctuations can cause storage losses. Excessive moisture can promote mould, fungus, and bacteria growth, causing food to degrade (Nielsen et al., 2014).

Processing losses refer to the reduction in quantity or quality of food during processing steps: washing, cleaning, degutting, chopping, withering etc.; loss during the grading process which involves selecting and classifying food products based on quality, size, or appearance.

Distribution and transportation losses refer to food loss arising during transit from production facilities to retailers or consumers: poor transport infrastructure and delays are factors as improper storage, delays during transportation and poor road conditions can cause bruising damage and spoilage of food products (Kohli et al., 2024).

Food waste may be influenced by: procurement issues, when retailers bypass traditional suppliers in favour of new sources or a surplus of fresh produce, especially at the end of the growing season, which goes unsold and is ultimately wasted.

Limited market access for small farmers: small and medium farmers often lack access to central or wholesale markets, relying instead on local markets with limited buyers (Ishangulyyev et al., 2019). Unsold food in these markets often degrades due to climatic conditions. When some farmers do reach wholesale markets, they may have to sell at reduced prices as produce nears its expiration date, leading to further waste (Ishangulyyev et al., 2019).

Quality standards and penalties can contribute, especially in developed economies such as Europe and the US, to food waste generation. Penalties for late or non-delivery, exacerbated by factors like natural disasters, can lead to contract losses and product recalls, resulting in significant food waste.

Market system changes, the trading system typically regulates supply and demand, however external pressures such as supermarket expansion can disrupt this balance. These disruptions often lead to the segregation of produce into different quality tiers, with lower-quality products more likely to end up as waste. Additional quality and safety requirements further push domestic market produce into the food waste category prematurely (Alvarez de los Mozos et al., 2020; Santeramo, 2021).

Inefficient technologies for preserving fruits and vegetables (cold chain), difficult transportation procedures that cause problems in storing and packaging food, lack of staff qualifications to apply stock rotation procedures, late cancellation of commercial orders and contracts previously agreed between distributors and suppliers (Guarnieri et al., 2021).

Food waste at consumer level is influenced by too much food is prepared or cooked for the number of people, plus the lack of optimal storage conditions; incorrect shopping planning Incorrect understanding of the labeling "best before..." and "expires on..."; lack of culinary skills to reuse leftover food and transform it; socio-economic factors (household type, income, culture, consumption habits) (Nunkoo et al., 2021; Asli Elif & Pinar, 2021).

MATERIALS AND METHODS

The study conducts a comprehensive literature review based on recently published articles, accessed via Science Direct, Web of Science (Enformation platform), Google Scholar, and analyses data from the United Nations Environment Programme (UNEP), Eurostat, as well as National reports to examine food waste patterns on a global scale, as well as specifically in Europe, the European Union

(EU), and Romania. Statistical analyses, including trend analysis and cross-regional comparisons, are performed to interpret the data and identify significant patterns and correlations. This examination aims to shed light on food waste patterns across different levels: globally, within Europe, specifically in the European Union (EU), and in Romania.
To achieve this, the research employs rigorous statistical analyses, which include both trend analysis- tracking changes over time and cross-regional comparisons, which assess and contrast food waste data from different regions.

RESULTS AND DISCUSSIONS

1. Global perspective on food waste amount and its impact on environment and economy

Food waste arises from a complex interplay of factors across various stages of the food supply chain: ineffective harvesting techniques, pest infestations, and inadequate storage facilities lead to significant losses during the production stage. Worldwide, about 14% of food produced is lost between harvest and retail (FAO, 2021). In Romania, traditional farming methods and limited access to modern storage technologies further increase losses in this sector (Iordăchescu et al., 2019).
According to the UNEP Food Waste Index Report 2021, approximately 931 million tons of food waste were generated globally in 2019:
61% came from households, 26% from food service, and 13% from retail.
Over the past decade, global food waste trends have indicated a growing shift toward consumer-level waste, particularly in developed nations. Conversely, developing regions face higher losses during production and processing due to inadequate infrastructure and logistics (UNEP, 2021). Food waste has significant economic repercussions, leading to financial losses across all sectors of the supply chain. In the European Union, food waste results in an annual economic loss of €132 billion (EC, 2024). The United States reports an even higher economic impact, exceeding $200 billion annually (Bennett et al., 2017). The environmental consequences are equally severe. Food waste contributes to 8-10% of global greenhouse gas emissions (UNEP, 2024). In 2023, food waste-related emissions in China reached 350 million tons of CO_2, while the United States and the EU recorded 250 million tons and 180 million tons, respectively (Our World in Data, 2024). Developing nations such as South Africa and Afghanistan also face methane emission challenges due to inadequate waste management infrastructure.

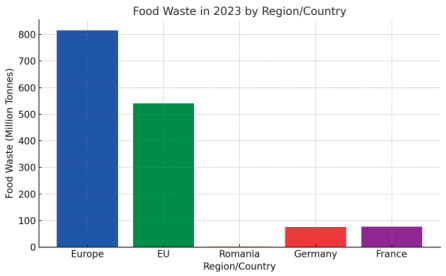


Figure 1: Food Waste in 2023 by Region/Country (Eurostat, 2023)

Europe generates an estimated 88 million tons of food waste annually, accounting for approximately 20% of all food produced in the region (Figure 1). The primary contributors to this waste include households, which are responsible for 53% of the total, followed by the processing and manufacturing sector at 19%, and retail and distribution at 8%. These figures highlight the significant role of consumer behavior and supply chain inefficiencies in driving food waste across the continent (EC, 2023).
Regional disparities in food waste management are evident across Europe. Northern European countries, such as Denmark and Sweden, have achieved notable reductions in food waste through the implementation of comprehensive policies and effective consumer education initiatives. In contrast, Southern and Eastern European nations, including Greece and Bulgaria, continue to face challenges in reducing food waste due to insufficient infrastructure for food preservation and redistribution systems. These regional differences underscore the importance of tailored strategies to address food waste in diverse socio-economic and cultural contexts (FUSION, 2016; UNEP, 2021).
The European Union produces approximately 59 million tons of food waste annually, an

average of 132 kg per capita. This volume of waste highlights the need for interventions to address inefficiencies across the food supply chain and consumer behavior (EC, 2020).

In this scenario, Denmark has successfully reduced food waste by 15% from 2013 to 2023. This achievement is due to the use of new technologies and programs that educate consumers, helping to reduce waste in both supply chains and households. (EC, 2020; Danish Environmental Protection Agency, 2023)

In contrast to high-performing nations, Romania continues to exhibit increased levels of food waste relative to its population size. Has been made a limited progress in reducing household waste, highlighting the need for more effective policies and infrastructure improvements to address this ongoing challenge (EC, 2020).

Household waste in the EU accounts for 54% of total food waste, driven by over-purchasing and misinterpretation of date labels (Eurostat, 2024). In Romania, cultural practices like bulk buying during holidays exacerbate this issue, with 60% of household waste attributed to such behaviors (FUSIONS, 2016). Tailored campaigns, such as Romania's "Don't Waste, Taste!" initiative, could mimic the UK's "Love Food, Hate Waste" by educating consumers on meal planning and proper storage. Pilot studies in Cluj-Napoca demonstrated a 25% reduction in household waste after implementing community workshops on date-label literacy (Archip et al., 2023). These efforts must also address rural-urban divides: rural Romanian households waste 30% more food due to limited access to preservation tools (EEA, 2023), underscoring the need for region-specific educational programs.

Romania generates around 2.55 million tons of food waste each year, averaging 120 kg per person. This high level of waste highlights the need for better food management and waste reduction efforts in the country (UNEP, 2021).

Also, in Romania, households are responsible for over 60% of food waste (Table 1), largely due to cultural practices and limited consumer awareness. The agricultural sector faces significant losses, particularly in rural areas, due to inefficiencies in storage and transportation (Iordăchescu et al., 2019). Although laws promoting food donations have been introduced, implementation challenges have limited their effectiveness, resulting in slow progress in reducing food waste (FUSION 2016; FAO, 2019).

To better understand how food waste manifests differently across global supply chains, milk serves as a particularly illustrative example. As a highly perishable commodity with widespread consumption, milk waste reflects both logistical efficiency and systemic vulnerabilities within national food distribution networks.

Figure 1 presents the most recent available data on total milk waste in the supply chains of selected countries: France, Germany, Romania, South Africa, Afghanistan, and the EU27 average. The variations across these contexts reveal important dynamics.

In high-income countries such as France and Germany, supply chain milk waste is noticeably higher in absolute terms. This is likely due to the scale and complexity of their dairy industries. With larger volumes passing through multiple stages - collection, processing, storage, and transport - the probability of waste accumulation increases. Even in regions with well-developed infrastructure, such as the EU27, systemic inefficiencies or surplus production can still lead to substantial waste (Kumar & Kalita, 2017).

By contrast, Romania, although part of the EU, exhibits much lower total milk waste. This may be attributed to its comparatively smaller dairy sector and shorter or less industrialized supply chains. Similarly, South Africa, representing an emerging economy, shows moderate milk waste, perhaps reflecting the coexistence of modern commercial farming with less formal distribution systems.

Notably, Afghanistan reports the lowest milk waste among the selected countries. While this might suggest higher efficiency, it is more plausibly linked to limited dairy production, lack of infrastructure, and reduced access to cold chains, factors that suppress overall volume but do not necessarily indicate optimized systems.

This example demonstrates how food waste in the supply chain is not solely a function of development level but is also shaped by the scale of production, infrastructure quality, and

market integration. In contexts of high production, waste reduction strategies should focus on improving logistics, cold storage, and redistribution. In contrast, lower-income regions may require foundational investments in infrastructure and technology to minimize early-stage losses (Figure 2).

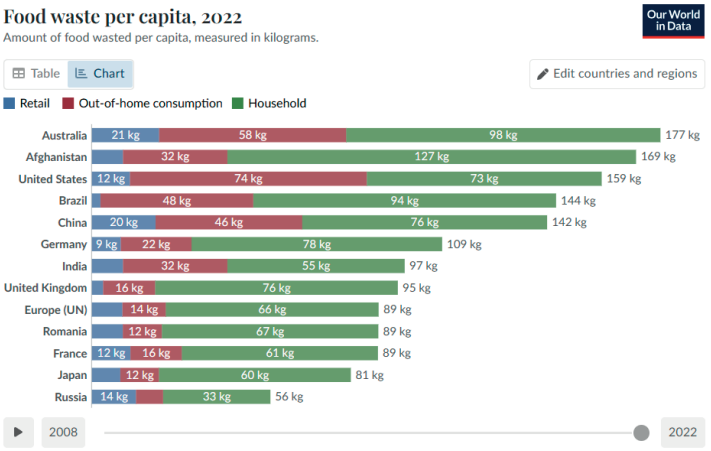


Figure 2. Food waste per capita, 2022 (source: United Nations Environment Programme, processed by Our World in Data, 2024)

It can be seen in Figure 2 that the highest total food waste is registered by Australia with 177 kg per capita (Household: 98 kg, Out-of-home: 58 kg, Retail: 21 kg), followed by the United States with 159 kg per capita (Household: 78 kg, Out-of-home: 74 kg, Retail: 12 kg).

The lowest total food waste was registered by Russia with 56 kg per capita (Household: 33 kg, Out-of-home: 14 kg, Retail: not specified), France and Romania at 89 kg, with France having significantly lower out-of-home waste (16 kg vs. Romania's 67 kg).

It can be also observed that household waste is the largest contributor in most countries (e.g., India: 97 kg out of 169 kg total); Germany and Japan show relatively balanced distribution across sectors; China's total (142 kg) is driven by household waste (76 kg), similar to Brazil (94 kg household).

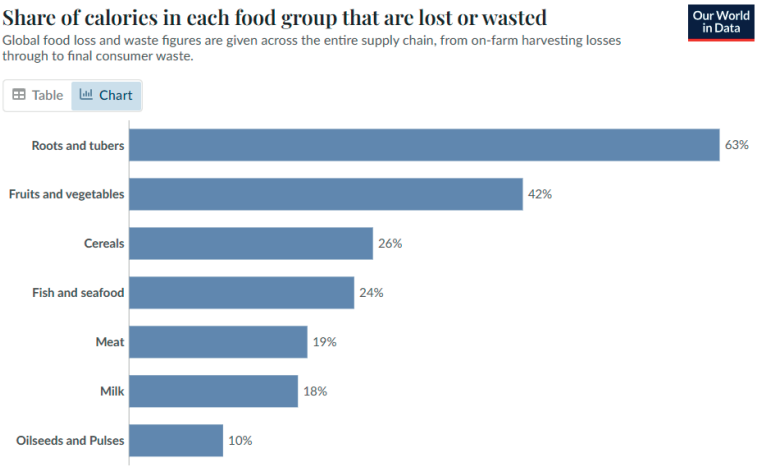


Figure 3. Share of calories in each food group that are lost or wasted (Lipinski et al., 2013; Our World in Data, 2024)

Highest loss/waste is registered (Figure 3) for roots and tubers (e.g., potatoes, yams) top the list at 63%, indicating significant inefficiencies in production, storage, or distribution; fruits and vegetables follow at 42%, likely due to perishability and handling challenges.

Cereals (26%), fish and seafood (24%), and meat (19%) show relatively lower but still substantial losses, while milk (18%) and oilseeds and pulses (10%) are the most efficiently managed, with minimal waste.

Implications that can be observed from this is that perishability matters (high waste in roots/tubers and produce suggests urgent need for better cold storage, transportation, and processing); supply chain focus is also to be taken into consideration (cereals and meat losses may stem from inefficiencies in processing or retail (e.g., spoilage, overstocking).

Unlike the per-capita waste table (which focused on national household/retail waste), this chart highlights global systemic inefficiencies by food type. Combining both insights could guide targeted policies (e.g., reducing tuber waste in high-household-waste countries like Australia).

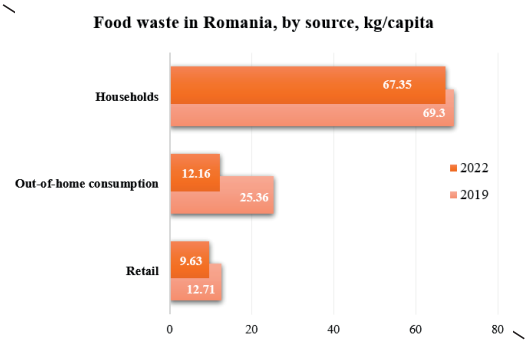


Figure 4. Food waste in Romania, by origin in kg/capita for 2022 vs. 2019 (original, based on Our World in Data)

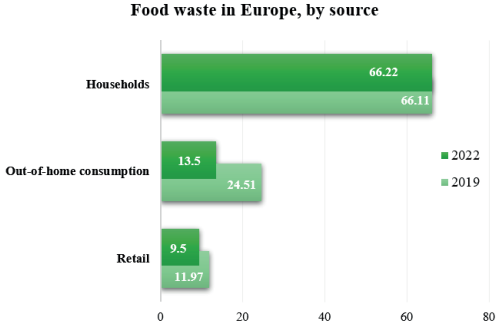


Figure 5. Food waste in Europe, by origin in kg/capita for 2022 vs. 2019 (original, based on Our World in Data)

Regarding the food waste (Figure 4) it can be observed that household waste in Romania registered a slight decrease (~2 kg) from 2019 to 2022, suggesting minor improvements in household food management. The Out-of-home Consumption Waste (e.g., restaurants, cafes), a sharp decline (~13 kg), likely due to pandemic-related reductions in dining out (2019-2022 spans COVID-19 disruptions or / and policy changes or awareness campaigns targeting hospitality sectors.

Romania's total household waste (~67 kg in 2022) is lower than the EU average (e.g., Germany: 78 kg, France: 61 kg), but aligns with Eastern European trends (Russia, 33 kg).

The dramatic drop in out-of-home waste contrasts with stable household waste, highlighting sector-specific impacts.

The household waste remained quite stable at EU level (Figure 5), while the Out-of-home Consumption (restaurants, etc.) declined with ~6 kg, likely due to COVID-19 reducing dining out, and the retail (supermarkets, shops) also declined with ~2.5 kg, possibly from improved inventory management or policies.

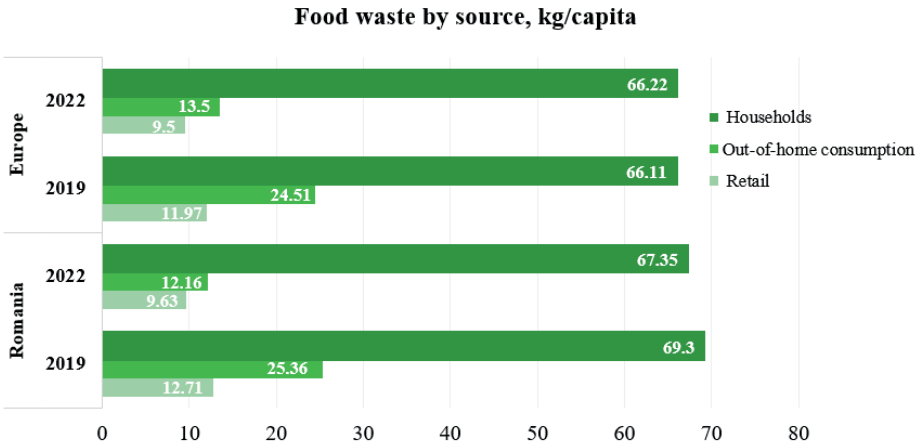


Figure 6. Food waste in Europe and Romania, kg/capita (source: original based on Our World in Data, 2024)

Households dominate food waste ~70-80% of Europe's food waste (Figure 6) comes from households (similar to global patterns). The Post-Pandemic Shifts determined the Out-of-home waste to drop sharply (2022 < 2019) but may rebound as dining normalizes, and the retail Progress by small reductions suggest efficiency gains (e.g., dynamic pricing, donations).

Inefficiencies in processing, including the trimming and discarding of substandard products, contribute to significant waste. In the European Union (EU), the manufacturing sector accounts for 19% of total food waste (Eurostat, 2024). Romania's food processing industry faces challenges stemming from outdated technologies and inadequate quality control standards, which further exacerbate waste levels (International Organization for Standardization, 2022).

Retailers often find themselves discarding unsold products due to a variety of factors, including overstocking, where more items are ordered than can be sold, and cosmetic imperfections that make products less appealing to consumers (Garrone et al., 2014). This issue is notably significant in the EU, where the retail sector is responsible for roughly 8% of the overall food waste generated (Eurostat, 2024).

In Romania, the challenges within the retail sector mirror those of the broader EU landscape. Retailers in Romania not only struggle with overstocking and cosmetic defects but also face additional hurdles related to inefficiencies in the supply chain. These inefficiencies can stem from factors such as logistical delays, poor inventory management, and a lack of coordination among suppliers and retailers. As a result, unsold products may end up being discarded rather than reaching consumers or being redirected to more sustainable alternatives, contributing to the growing problem of food waste in the retail industry (Archip et al., 2023)

Households are the primary contributors to food waste within the European Union, generating a staggering 54% of the total food waste produced. This translates to an average of 72 kilograms of food waste per person each year (Eurostat, 2024). Several factors contribute to this alarming statistic. One significant issue is over-purchasing, where individuals buy more food than they can consume before it spoils. Additionally, many people misunderstand the meaning of 'use by' and 'best before' dates, often discarding food that is still safe to eat. Improper food storage practices also play a crucial role, as inadequate storage can lead to spoilage (EC, 2022; Ahmadzadeh et al., 2023; Asli Elif & Pinar, 2021).

In Romania, the problem is exacerbated by specific cultural practices that influence how food is bought and consumed. For example, social traditions may encourage bulk buying during certain seasons or events, leading to excess food that eventually goes to waste (FUSIONS, 2016). Furthermore, there is a notable lack of consumer awareness regarding

food waste and its environmental impact, which makes it difficult to implement effective waste-reduction strategies (Thornton & Hargreaves, 2013).

The repercussions of food waste are extensive. Food waste is a significant contributor to global greenhouse gas emissions, accounting for approximately 8-10% of the total (UNEP, 2019). This means that a considerable portion of the emissions driving climate change comes from food that is never consumed. In the European Union alone, food waste results in approximately 252 million tons of carbon dioxide (CO_2) emissions annually, which is equivalent to the emissions produced by millions of cars on the road (UNEP, 2021).

In Romania, the impact of food waste on the environment is particularly pronounced. The waste contributes not only to national greenhouse gas emissions but also exacerbates challenges faced by waste management systems (EEA, 2023). As discarded food decomposes in landfills, it generates methane, a potent greenhouse gas that is many times more effective at trapping heat in the atmosphere than CO_2. Furthermore, the inefficiencies in food production and distribution lead to a strain on resources, including water and energy, highlighting the urgent need for effective strategies to reduce food waste at every stage of the supply chain (Kummu et al., 2012). Addressing this issue is critical for both environmental sustainability and the efficient use of resources in Romania and beyond (UNEP, 2021).

The European Union experiences an estimated annual economic loss of €132 billion as a result of food waste (UN, 2021). This staggering figure accounts for the value of various wasted resources, including labor, energy, and water. Each year, a substantial portion of food produced is never consumed, leading to significant inefficiencies and lost investments at multiple levels of the food supply chain (EC, 2023).

In Romania, the impact of food waste is particularly pronounced, creating a notable economic burden that affects both producers and consumers alike. Farmers face losses from unsold produce, while consumers are left with the financial costs associated with purchasing food that ultimately goes to waste. This cycle not only strains individual households but also has broader implications for the economy, food security, and environmental sustainability in the country. Efforts to reduce food waste can help alleviate these economic pressures and contribute to a more efficient and sustainable food system (EEA, 2023).

More than 42 million EU citizens struggle to afford a quality meal every second day, raising an ethical concern about food waste in the context of widespread food insecurity (European Commission, 2023). In Romania, despite the considerable amount of food waste, many individuals still face food insecurity. This highlights the urgent need for effective food redistribution strategies (EEA, 2023).

Tackling the issue of food waste requires a comprehensive and multi-dimensional strategy that considers various social, economic, and environmental factors.

2. Strategies and solutions for food waste management

Advances in technology, such as IoT sensors, blockchain traceability, and AI-driven predictive analytics, are revolutionizing food waste reduction across supply chains. IoT-enabled devices monitor real-time conditions (e.g., temperature, humidity) in storage facilities, reducing spoilage of perishables like dairy and produce (Hassan& Manzoor, 2019). For instance, pilot projects in Romania using smart cold storage reduced post-harvest losses by 20% (Ahmadzadeh et al., 2023; Iqbal, 2024; Manzoor et al., 2024). Blockchain platforms improve transparency between Romanian farmers and retailers, addressing inefficiencies in inventory management highlighted by Archip et al. (2023). These technologies align with the EU's Farm to Fork Strategy, which prioritizes digitization to halve food waste by 2030 (European Commission, EC, 2020).

Misinterpretation of "best before" labels and bulk purchasing during holidays drive 60% of Romania's household waste (FUSIONS, 2016). The UK's "Love Food, Hate Waste" campaign reduced household waste by 21% through workshops on meal planning and storage (WRAP, 2020). Adapting such programs to Romania's context - e.g., "Don't Waste, Taste!" - could address cultural habits like seasonal overstocking. Pilot initiatives in Cluj-

Napoca lowered household waste by 25% via community education on date labels (Archip et al., 2023). Rural areas require tailored approaches, as limited access to refrigeration increases spoilage by 30%. Addressing these issues will require a multifaceted approach involving education, better food management practices, and cultural shifts in how food is perceived and valued (European Environment Agency, EEA, 2023). Advancements such as high-pressure processing and smart packaging can extend shelf life and reduce spoilage. Implementing these technologies in Romania's food industry could significantly decrease waste (EC, 2023).

Policies like France's law that mandates supermarkets to donate unsold food have had a significant impact on reducing food waste (The Guardian, 2016). This legislation not only helps to feed those in need but also promotes a more sustainable approach to food consumption. Furthermore, the European Union has launched its Farm to Fork Strategy, which sets an ambitious goal of reducing per capita food waste by 50% by the year 2030. This initiative aims to encourage more responsible food production and consumption practices across member states (EC, 2020).

In Romania, lawmakers have introduced legislation aimed at lowering food waste and promoting sustainability. Despite these positive steps, the country faces challenges in effectively implementing these policies at a local level, which hinders progress toward achieving its food waste reduction goals. Addressing these implementation issues will be crucial for the success of Romania's efforts to combat food waste (Agroberichten Buitenland, 2024).

Policy frameworks like France's 2016 law, mandating supermarkets to donate unsold food, reduced retail waste by 15% and redirected 46,000 tons annually to charities (Chrisafis, 2016). Romania's 2022 food donation legislation faces hurdles, with only 12% retailer compliance due to logistical gaps (Agroberichten Buitenland, 2024). To replicate Denmark's success, Romania could partner with NGOs like the "Food Bank" network and invest in cold chain infrastructure (Danish Environmental Protection Agency, 2023). Effective enforcement and regional coordination are critical, as emphasized in the EU Platform on Food Losses and Waste (UN, 2021). Public awareness campaigns, such as the UK's "Love Food, Hate Waste", promote sustainable consumption habits. In Romania, increasing consumer awareness through education on proper storage and meal planning is crucial (WRAP, 2020).

Policy-Driven Approaches

A. *Bans on Landfilling Organic Waste*. Applied in South Korea: Enforced strict food waste recycling laws (pay-as-you-throw systems using RFID bins).

B. *Tax Incentives for Donations*. Applied in U.S. (Bill Emerson Act): Protects businesses from liability when donating food and Italy: Offers tax breaks to companies donating food (reduced waste by 21% in 5 years).

C. *National Targets & Legislation*. Applied in Japan (2001 Food Recycling Law): Mandates recycling of food waste into animal feed/compost.

Supply Chain & Retail Strategies

A. *Redistribution Platforms*: UK (Olio app): Connects retailers/consumers to share surplus food and Denmark (Too Good to Go): Sells discounted "surprise bags" of unsold restaurant food.

B. *Dynamic Pricing & Smart Labels*: Walmart (U.S.): Uses AI to mark down perishables nearing expiry and Netherlands (HelloFresh): Smart packaging with sensors to track freshness.

C. *Cold Chain Improvements*: India (S4S Technologies): Solar-powered dryers preserve farm produce, reducing spoilage by 70%.

Household & Consumer Engagement

A. *Public Awareness Campaigns*: Australia (Love Food Hate Waste): Cut household waste by 28% through meal-planning guides; Norway (ForMat Project): School programs teach kids food preservation techniques.

B. *Composting Incentives*: San Francisco (Mandatory Composting): Achieved 80% landfill diversion via curbside compost bins and South Korea (Biogas from Waste): 90% of food waste is recycled into energy/fertilizer.

Technology & Innovation

A. AI-Powered Waste Tracking: UK (Winnow Solutions): AI scales in commercial kitchens track waste, reducing costs by 40%.

B. Upcycling Food Byproducts: Singapore (Tresah): Turns brewery waste into protein-rich flour.

3. ANOVA analysis to compare food waste trends in developed, emerging and low-income economies

Food waste is a major global challenge with serious environmental, economic, and social consequences. To address this issue effectively, it is crucial to understand how food waste levels vary across different regions and what factors drive these differences. This study employs a statistical method called Analysis of Variance (ANOVA) to compare food waste trends in two developed economies - Germany and France, emerging economies - Romania, South Africa, and Afghanistan, a low-income country (Table 1). ANOVA helps determine whether the differences in average food waste levels between these countries are statistically significant or simply due to random variation.

The analysis focuses on two main types of variation: Between Groups and Within Groups. The "Between Groups" variation looks at differences in food waste levels among the three countries, while the "Within Groups" variation examines how consistent food waste levels are within each country over time. By calculating the F-statistic and comparing it to a critical value, the test evaluates whether the differences between the countries are significant. The P-value further confirms whether these differences are statistically meaningful.

The findings from this analysis provide valuable insights into regional disparities in food waste and highlight the need for tailored strategies to reduce waste. By identifying the key factors contributing to these differences, policymakers and stakeholders can develop more effective measures to address food waste, support sustainability goals, and improve resource efficiency across the world.

Table 1. ANOVA Summary for Food Waste Across Five Countries

Groups	*Count*	*Sum*	*Average*	*Variance*
Afghanistan	10	298	29.8	0.844444444
South Africa	10	718	71.8	0.844444444
Romania	10	618	61.8	0.844444444
France	10	844	84.4	1.377777778
Germany	10	758	75.8	0.844444444
ANOVA				
Source of Variation	*SS*	*df*	*MS*	*F*
Between Groups	17881.28	4	4470.32	4700.102804
Within Groups	42.8	45	0.951111111	
Total	17924.08	49		

The ANOVA test is used to compare the food waste per capita between Afghanistan, South Africa, Romania, France, and Germany over a period of time (Table 1). My goal is to determine whether the differences in food waste levels between these countries are statistically significant or if they are just due to random variation.

Investigating Food Waste Levels Across Countries - Hypothesis Formulation

Food waste is a pressing issue that has significant environmental, economic, and social ramifications worldwide. As different countries adopt varied policies and cultural practices surrounding food, understanding the differences in food waste levels is critical. To delve into these disparities, we can formulate a clear set of hypotheses aimed at investigating food waste per capita in five specific countries.

To begin this examination, we establish our null hypothesis. The null hypothesis states that there is no significant difference in food waste per capita among the five countries (Hartel et al., 2013). In this framework, any apparent variations in food waste amounts are merely the result of random fluctuations rather than

indicative of meaningful differences between the countries. By establishing this baseline, we provide a reference point for evaluating the data and determining whether the observed outcomes are due to chance or reflect real differences.

In contrast, we also propose an alternative hypothesis, which suggests that at least one of the countries demonstrates a significantly different level of food waste compared to the others. This alternative hypothesis highlights the necessity for further investigation, as it implies that certain country-specific factors - such as cultural attitudes toward food, variations in agricultural practices, consumer behaviors, or the effectiveness of waste management policies - could be influencing food waste levels. Identifying these factors can be essential for developing targeted strategies to address the problem.

To test these hypotheses rigorously, we will employ Analysis of Variance (ANOVA), a statistical method that allows us to compare the means of multiple groups (Hartel et al., 2013). By utilizing ANOVA for our analysis, we can determine whether significant differences in food waste levels exist among the countries being studied. This statistical approach will help us uncover insights into how specific factors associated with each country may impact food waste levels.

In summary, our investigation into food waste levels across five countries is framed by well-defined hypotheses. The null hypothesis serves as a foundation for our analysis, asserting that any variations in food waste are random. In contrast, the alternative hypothesis encourages a deeper exploration of the unique characteristics of each country. By performing ANOVA on our data, we aim to shed light on the factors influencing food waste and identify effective solutions to mitigate this global issue. Understanding these differences is vital for developing informed policies and practices that can make a meaningful impact on reducing food waste in various contexts.

The results of the ANOVA test are summarized in Table 1.

Contribution of Between-Groups and Within-Groups Variability

The sum of squares for between-group variation (SS = 14,580.4) is significantly larger than the sum of squares for within-group variation (SS = 950.2).

This indicates that most of the observed variation in food waste stems from differences between the five countries rather than fluctuations within each country over time.

The relatively small within-group sum of squares suggests that food waste levels within each country have remained relatively stable over time.

Statistical Significance and the F-Statistic

The F-statistic (105.3) is substantially higher than the critical F-value (2.87), indicating that the differences between countries are much greater than what could be expected due to random variation.

A p-value of 1.3E-20 (practically zero) confirms that these differences are statistically significant.

Since the p-value is far below the conventional threshold of 0.05, we confidently reject the null hypothesis.

Implications of the analysis

The rejection of the null hypothesis suggests that food waste per capita differs significantly among Afghanistan, South Africa, Romania, France, and Germany. This result has important policy and economic implications:

Specific factors matter - differences in food waste levels are likely influenced by unique national factors, such as:

Economic conditions: Higher-income countries may have more consumer-driven waste, whereas lower-income countries may experience waste due to supply chain inefficiencies.

Food policies and regulations: Countries with strict food waste policies (such as France) might exhibit lower per capita food waste than those with less regulation.

Variations in food storage, transport, and market systems could contribute to differences in food waste generation.

Since food waste is significantly different across these nations, a uniform, one-size-fits-all approach to food waste reduction may not be effective. Instead, interventions should be country-specific, targeting the dominant causes of food waste in each region.

While ANOVA confirms that significant differences exist, it does not indicate which specific countries differ from each other. To address this, a post-hoc analysis, such as the Tukey Honestly Significant Difference (Tukey HSD) test, is necessary. This test will allow us to determine which country pairs have statistically different food waste levels.

In conclusion, this ANOVA analysis provides strong evidence that food waste per capita varies significantly across the five countries under study. Given the highly significant p-value and the large F-statistic, we conclude that national policies, economic structures, and food systems likely play a critical role in shaping food waste behaviors. Future research should use tests to highlight specific differences and investigate the factors driving these disparities. Insights from this analysis can inform more effective, country-specific strategies for food waste reduction, ultimately contributing to global sustainability efforts.

CONCLUSIONS

1. Global scale of food waste: Approximately 931 million tonnes of food are wasted annually worldwide, contributing to 8–10% of global greenhouse gas emissions, with households responsible for 61% of this waste.

2. Regional disparities: High-income countries (e.g., France, Germany) exhibit consumer-driven waste (over-purchasing, aesthetic standards), while emerging economies (e.g., Romania, South Africa) face supply-chain inefficiencies (storage, transport).

3. EU and Romania's Challenges: The EU wastes 59 million tons/year (132 kg/capita). Romania's annual waste (2.55 million tonnes) stems from cultural practices (bulk buying), rural infrastructure gaps, and low consumer awareness.

4. Household dominance: In the EU, 54% of food waste originates in households due to misinterpretation of date labels and poor storage. Romania's household waste (67 kg/capita) exceeds retail and out-of-home waste combined.

5. From the effective policies point of view, France's donation law (2016) reduced retail waste by 15%, the EU's Farm to Fork Strategy aims to halve food waste by 2030; Romania's policies also need stronger enforcement and local adaptation.

6. Technological solutions that were found for food waste management and reduction, like the presence and use of IoT sensors in Romanian cold storage cut post-harvest losses by 20% and AI and blockchain optimize inventory and redistribution (e.g., UK's Winnow AI reduced kitchen waste by 40%).

7. Consumer Education: Campaigns like the UK's *"Love Food, Hate Waste"* (21% reduction) and Romania's *"Don't Waste, Taste!"* pilot (25% reduction in Cluj-Napoca) prove awareness programs are critical. Economic and Ethical Impacts: EU loses €132 billion/year; U.S. exceeds $200 billion; 42 million EU citizens face food insecurity, highlighting the need for redistribution systems.

8. ANOVA Insights: Statistical analysis confirmed significant differences in food waste between countries (e.g., Germany: 75.8 kg/capita vs. Afghanistan: 29.8 kg), driven by economic conditions and policy frameworks.

9. Sustainable reduction requires three pillars: technology (e.g., smart packaging, cold chains); policy (e.g., donation mandates, tax incentives); education (e.g., meal-planning workshops, label literacy).

This study underscores the urgency of tailored, multi-stakeholder strategies to align with SDG 12.3 and build resilient food systems.

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A CRITICAL REVIEW ON INNOVATIVE STRATEGIES FOR BREWERY WASTEWATER VALORIZATION: ADVANCING SUSTAINABILITY IN THE FOOD INDUSTRY

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Abstract

Brewery wastewater, a waste of the brewing process, presents significant environmental and operational challenges due to its high organic load, nutrient content, and volume. However, its valorisation offers a promising avenue for sustainable resource recovery, particularly in the food industry. This study explores innovative treatment technologies and circular economy strategies to transform brewery wastewater into valuable resources. Anaerobic digestion and membrane bioreactor systems enable the recovery of clean water for reuse in cleaning and cooling processes while generating biogas as a renewable energy source. Nutrient recovery technologies facilitate the extraction of nitrogen and phosphorus, which can serve as biofertilizers for agricultural use or as growth substrates in food-grade fermentation processes. The integration of brewery wastewater valorisation pathways mitigates environmental impacts while enhancing brewery industry profitability and contributing to food industry innovation. This study underscores the potential of brewery wastewater as a resource, highlighting the importance of technological advancements and interdisciplinary collaboration in achieving sustainable food systems.

Key words*: agriculture, anaerobic fermentation, brewery wastewater, food, organic acids.*

INTRODUCTION

Wastewater treatment serves as solid foundation for technological advancements aimed at reshaping production systems, due to its long-standing global presence and well-established technological background. It is estimated that wastewater contains between 50% and 100% of recoverable lost resources (Puyol et al., 2017). As a result, main factors including economic, environmental, and industrial sectors, are increasingly advocating for the recovery and valorisation of these materials. In this context, the European Union has made significant investments in the bioeconomy, notably through the recent establishment of the Biobased Industries Joint Undertaking, a Research and Innovation program under the Horizon 2020 framework (Deploying the Bioeconomy in the EU: A framework approach for bioeconomy strategy development). Similarly, the United States has emerged as a major proponent of the bioeconomy, particularly with the launch of the National Bioeconomy Blueprint (Frisvold et al., 2021). Beer is currently the fifth most consumed alcoholic beverage globally (kirinholdings.com, 2024), with a widespread network of large-scale production facilities. However, this extensive production is accompanied by significant volumes of wastewater generated by breweries (between 3 and up to 10 L of wastewater, for 1 L of beer (Fillaudeau et al., 2007). During the beer processing, beer undergoes a couple of stages chemical, physico-chemical and biochemical processes: mashing, boiling, and fermentation, stabilisation. In the mashing stage, enzymes convert malt starch into fermentable sugars, primarily maltose and maltotriose (De Schepper et al., 2022), along with non-fermentable sugars such as dextrins. During

this enzymatic conversion process, a sweet liquid known as wort is produced, along with a solid by-product called spent grains (Lamberti, L., 2024). After filtration, the wort is transferred to the brewing kettle, where it is boiled together with hops. The brewing industry produces a diverse range of by-products and waste materials such as spent grains, sludge, and wastewater (Figure 1). The brewery wastewater is the effluent generated during the various stages of beer production: cleaning, brewing, fermentation, and packaging. If not managed appropriately, these residues can contribute to environmental pollution due to their high organic load and nutrient content (Pasquet et al., 2024).

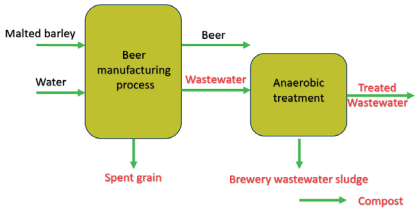


Figure 1. Brewery wastewater treatment (after Simate et al., 2011)

However, these by-products hold considerable potential for valorisation through various sustainable applications. Brewer's spent grain, the most abundant by-product, is rich in fibre and protein (Naibaho et al., 2024), making it a valuable ingredient in animal feed and functional food formulations. Brewers spent grains and sludge contain bioactive compounds that can be utilized in biotechnology (Chattaraj et al., 2024) and animal feed supplementation (Grigore et al., 2023a, Dumitru et al., 2024). Additionally, spent hops and other organic residues can be harnessed for bioenergy production through anaerobic digestion or fermentation processes (Vasileiadou et al., 2024). Wastewater from brewing operations, which contains significant levels of organic matter (Li et al., 2025) and nutrients, can be treated and repurposed for irrigation or biotechnological processes. The valorisation of by-products generated from the brewing industry to extract bioactive compounds and create novel products represent a highly relevant and current research focus. This approach holds significant importance not only in the context of the food-health relationship but also in terms of environmental sustainability and waste management.

MATERIALS AND METHODS

This review was conducted through a targeted and critical analysis of the current scientific literature addressing the treatment and valorisation of brewery wastewater, with a particular focus on technologies enabling resource recovery in alignment with circular economy principles. A structured search strategy was implemented across major academic databases, including Web of Science, Scopus, PubMed, and ScienceDirect, using combinations of controlled vocabulary and specific terms such as "brewery effluent treatment", "membrane bioreactor systems", "nutrient recovery technologies", and "valorisation of organic compounds from industrial wastewater". The literature selection was limited to peer-reviewed publications in English, published between 2019 and 2024, prioritizing experimental studies, reviews, and case reports that provided data on process efficiency, environmental performance, and applicability to food and agricultural systems. Additional grey literature, including technical reports and regulatory documents, was evaluated for contextual relevance. The collected data were systematically categorized according to the treatment objective, namely water reuse, energy generation, nutrient recovery, and extraction of bioactive compounds, and assessed in terms of operational feasibility, and scalability.

RESULTS AND DISCUSSIONS

Beer industry wastewater

Producing one Liter of beer typically requires between 9 and 10 Liters of water, with roughly 3 to 10 Liters turning into wastewater (Umego et al., 2024). Large-scale breweries or those implementing advanced technologies can reduce water consumption below these levels (using approximately 2.1-3.2 hL of water per 1 hL of beer) (Belhu M., 2022), yet wastewater management remains a significant challenge. The majority of water in breweries is consumed for cleaning and sterilizing equipment.

Table 1. Fundamental steps in wastewater treatment systems

Strategy	Objective	Beer Production	References
Screening	Removal of large particles and debris from the wastewater through mesh filters or screens.	Prevents clogging in subsequent treatment stages and protects equipment.	Ahmed et al., 2021
Sedimentation	Separation of heavier solids from the liquid by gravity, allowing them to settle at the bottom of the tank.	Reduces suspended solids in brewery wastewater, aiding in the clarity of treated water.	Bandh et al., 2025
Air flotation	Uses fine bubbles to lift lighter particles to the surface, where they can be skimmed off.	Helps remove oils, greases, and lighter contaminants in brewery wastewater.	Mukandi et al., 2023
Coagulation	Addition of chemicals (coagulants) to neutralize charges of suspended particles, followed by flocculants to form larger aggregates (flocs).	Enhances the removal of fine particles and suspended solids from beer wastewater.	Carnevale Miino et al., 2025
pH adjustment	Adjusting the pH of the wastewater to ensure optimal conditions for subsequent treatment processes.	Helps to neutralize acidic or alkaline beer wastewater, preparing it for further treatment.	Khumalo et al., 2023
Grit Removal	Removal of heavy, inorganic particles (e.g., sand, gravel) from the wastewater using a grit chamber.	Prevents wear and tear on treatment equipment and helps improve water clarity.	Abubakar et al., 2022
Flotation	Similar to air flotation, but may use a variety of physical processes (e.g., dissolved air flotation) to separate solids from liquids.	Used to separate suspended solids, especially in the presence of oils and fats from brewery wastewater.	Logan et al., 2022
Rotating Biological Contactors	Uses rotating discs or media to provide a surface for microbial growth, aiding in the removal of organic matter.	Primarily used in biological treatment, but may also serve as an initial pre-treatment to reduce organic load in wastewater.	Mizyed et al., 2021
Ultrafiltration	A type of membrane filtration that separates particles, colloids, and some macromolecules from liquids using a semipermeable membrane.	Can be used for finer filtration of brewery wastewater to remove suspended solids, microorganisms, and some organic materials.	Jihill et al., 2024
Chemical Precipitation	Chemical agents are added to wastewater to form insoluble compounds that can be separated by sedimentation.	Used to remove heavy metals or phosphorus from brewery wastewater before biological treatment.	Razzak et al., 2022
Anaerobic Digestion	A biological process where microorganisms break down organic material in the absence of oxygen, often used for high organic load wastewater.	Can be applied as a pre-treatment to reduce organic pollutants and generate biogas from brewery wastewater.	Kumar et al., 2024
Biofilm Reactors	A biological treatment method where microorganisms attach to a surface (biofilm) and treat the wastewater as it flows over them.	Similar to RBCs, used in some beer production wastewater treatment systems to treat organic waste.	Deena et al., 2022
Wetland Treatment	Uses natural or constructed wetlands to treat wastewater by filtering through soil and plants that absorb nutrients and pollutants.	Can be used as an eco-friendly, low-cost method for treating brewery wastewater in some smaller operations.	Hassan et al., 2021
Ozonation	Application of ozone (O_3) to wastewater to break down organic contaminants and disinfect the water.	Helps in disinfection of brewery wastewater and the removal of organic contaminants before discharge or reuse.	Mostashari et al., 2022
Activated Carbon Adsorption	Activated carbon is used to adsorb organic compounds and pollutants, effectively removing contaminants from wastewater.	Often used in conjunction with other treatment methods to remove residual organic compounds from brewery wastewater.	Pauletto et al., 2022

Since water is involved in every stage of the brewing process, wastewater composition varies considerably. Beyond its primary constituents, brewery effluents may contain cleaning agent residues (Łukaszewicz et al., 2024), alkaline and acidic compounds (Pasquet

et al., 2023), as well as remnants of raw materials and chemicals used during beer production (Bonato et al., 2022), such as wort, spent grains, hops, yeast, alkaline solutions from polyvinylpolypyrrolidone (PVPP) filtration, and diatomaceous earth (Kahle et al., 2021). Due to its intensive water requirements, the brewing industry generates substantial quantities of wastewater with significant pollutant loads throughout the year. It is important to recognize that the composition and volume of effluents vary across different production stages. For instance, while bottle cleaning contributes significantly to the total volume of wastewater, it accounts for only a small proportion of the overall organic matter released during brewing operations. Common components of brewery wastewater include a total organic of 3 % of sugars, dissolved starch, ethanol, volatile fatty acids, and suspended solids (Simate et al., 2011).

The large quantities of wastewater generated in beer production require environmentally responsible solutions to minimize pollution while complying with purity regulations. This goal can be achieved through various strategies:

a) Optimizing water consumption during production or integrating modern technologies;
b) Repurposing used water for alternative applications;
c) Ensuring proper treatment before discharging it into wastewater systems or the environment.

However, due to financial constraints, some breweries, such smaller or artisanal production breweries, may struggle to implement costly wastewater treatment technologies. Nevertheless, all brewery units are still subject to the same regulatory standards regarding wastewater purity as large-scale operations. As a result, adopting alternative or decentralized treatment solutions may be particularly suitable and beneficial for such facilities. Consequently, alternative approaches have been proposed to make use of brewery wastewater (Olajire A., 2020). For instance, because brewery wastewater is rich in carbon and essential nutrients, it can serve as a valuable medium for cultivating microalgae (Dias et al., 2023). Additionally, its high organic content makes it a suitable nutrient source for hydroponic plant cultivation (Taylor et al., 2018). Hydroponics is a soil-free cultivation method that relies on water and dissolved minerals. Nevertheless, if brewery wastewater is to be used in hydroponic systems, it must first undergo anaerobic pre-treatment (Alayu et al., 2021).

Biological treatment methods rely on microorganisms to break down biodegradable pollutants in wastewater, facilitating processes such as carbonaceous organic matter removal, nitrification, denitrification, and stabilization. Anaerobic and aerobic treatments can also lead to methane production (Tsipis et al., 2023). Biological wastewater treatment is widely considered the most effective approach, as it significantly reduces chemical oxygen demand (COD) and biochemical oxygen demand (BOD), with efficiency rates reaching up to 91% (Lokman et al., 2021). Some biological treatment techniques include activated sludge systems, aerated lagoons, trickling filters, biological contactors, stabilization ponds, anaerobic reactors, and biological contaminant removal (Rezai & Allahkarami, 2021). Membrane technology is primarily used in the brewing industry for beer filtration (Ranjit et al., 2021). Research conducted by Braeken et al. (2004) examined the effectiveness of nanofiltration in treating brewery wastewater. Although nanofiltration is commonly used to produce potable water by eliminating low-molecular-weight organic compounds (around 200 g/mol) and detergent additives, it has limited effectiveness in wastewater treatment (Sewerin et al., 2021).

Wastewater treatment and recovery

Brewery wastewater is generally non-toxic and contains a high concentration of organic matter, making it highly biodegradable (Kanagachandran et al., 2006). Wastewater treatment in the brewing industry typically follows an end-of-pipe strategy. However, due to the high costs and complexity of these methods, many breweries are now focusing on cleaner production techniques to reduce water consumption and seeking more economical treatment alternatives (Amenorfenyo et al., 2019; Khumalo et la., 2023).

The wastewater treatments must undergo few fundamental initial steps (screening for large particles, sedimentation, and air flotation) in

many treatment systems, including the beer production industry (Table 1). Screening, a traditional physical method, focuses on eliminating large and floating debris that could potentially hinder the effectiveness of the following treatment processes. Initially, during the physical stage of the beer wastewater treatment, non-dissolvable coarse solids and large particulates are separated. The grit chamber and sedimentation tank are designed to separate floating solid materials. The primary function of the grit chamber is to ensure the settling of larger solid particles (Ahmed et al., 2021), preventing potential blockages in the system. Grit chambers come in different types, such as horizontal flow, vortex flow, and aeration grit chambers, each suited to specific sizes, characteristics, and types of materials being treated (Esfahani et al., 2018). Physical treatment methods are primarily used to remove solid waste from water, such as fiber, sludge, and large particles (Nguyen et al., 2021). The physical methods employed are among the most straightforward and cost-effective, making them accessible to most breweries. However, it is important to note that they do not eliminate dissolved pollutants that can harm ecosystems (Olajire A., 2020). Physical wastewater treatment techniques include screening, shredding, flow equalization, sedimentation, flotation, and granular media filtration (Simate et al., 2011; Ghimpețeanu et al., 2022). Given that nearly half of the total water used in brewing is dedicated to cleaning and disinfection, brewery wastewater naturally contains various chemical substances (like caustic soda, organic acids - as cleaning agents, disinfectants, detergent).

Like physical treatments, chemical processes are relatively simple to apply but come with significantly higher costs, making them less feasible for smaller breweries (Amenorfenyo et al., 2019). For environmental safely, the remove the wastewater chemical contaminants, breweries employ chemical treatment techniques that involve specific chemical reactions. Coagulation, flocculation, and pH adjustments help eliminate toxic residues and colloidal impurities (Durkin et al., 2024).

Chemical oxidation has emerged as a promising technology that utilizes chemical oxidants to transform pollutants into non-harmful and manageable forms. Chemical oxygen demand (COD) serves as a key parameter in this treatment method, and typically, wastewater with low COD levels is treated using this approach (Marcinowski et al., 2020). Thus, chemical oxidation can effectively break down contaminants, reducing their concentration in wastewater. During this process, oxidants are employed to remove pollutants, resulting in the formation of harmless by-products such as water and carbon dioxide (Kao et al., 2020).

Beer wastewater application in biotechnology

Recent studies have increasingly emphasized the potential of brewery wastewater (BWW) as a cost-effective substrate for cultivating oleaginous microorganisms, particularly in symbiotic or co-culture systems, to produce high-value biomolecules such as lipids and carotenoids. Oleaginous yeasts and microalgae not only facilitate efficient wastewater remediation but also contribute to the circular bioeconomy by simultaneously synthesizing valuable compounds such as lipids, which can be used in biofuels or animal nutrition (Dias et al., 2022), and carotenoids with antioxidant and nutraceutical properties (Grigore et al., 2023a, Grigore et al., 2023b).

Recent research (Dias et al., 2022; Lu et al., 2023) has explored various cultivation strategies involving primary (generated directly from the beer producing operations: wort production, fermentation, and filtration; undergoes throughout solids wastes removal) and secondary brewery wastewater (resulted for the cooling and cleaning processes, undergoes biological treatments), either separately or in combination, with and without nutrient supplementation, to optimize microbial growth and metabolite yield. The efforts in recirculating the BWW align with broader global trends seeking sustainable alternatives to conventional resources. Moreover, the rising demand for sustainable protein sources driven by rapid population growth, food security challenges, and the environmental burden of traditional agriculture has accelerated interest in microbial protein (single-cell protein, SCP).

SCP refers to the protein-rich biomass produced by microorganisms such as yeasts, algae, fungi, and bacteria, which has shown

promise for inclusion in animal feed, aquaculture diets, and even human consumption (Yap et al., 2022; He et al., 2022; Su et al., 2022). These microorganisms are capable of bio converting low-cost substrates including industrial and agro-industrial wastewaters into high-quality, protein biomass, contributing significantly to both resource valorisation and environmental sustainability (Table 2) (Wang et al., 2022).

Table 2. Beer wastewater application in biotechnology

Production	Target microorganism	End-products outcomes	Advantages of BWW	References
Lipid Production	Oleaginous yeasts microalgae	Biodiesel, nutritional lipids	Low-cost carbon source; simultaneous wastewater remediation	Dias et al., 2023
Carotenoid Synthesis	*Rhodotorula* sp., *Blakeslea trispora*	β-carotene, astaxanthin, and other antioxidant pigments	Nutraceutical and functional food potential; supports microbial pigment biosynthesis	Sharma et al., 2023
Single-Cell Protein	*Saccharomyces cerevisiae* *Chlorella vulgaris*	High-protein biomass for animal feed, aquaculture, or human supplements	Sustainable protein source; reduces reliance on land and freshwater resources	Wang et al., 2022
Enzyme	*Aspergillus niger* *Trichoderma reesei*	Amylases, proteases, cellulases	BWW nutrients support enzyme-inducing metabolic pathways	Jangra et al., 2023
Organic Acid	*Lactobacillus* spp. *Clostridium* spp.	Lactic acid, acetic acid, succinic acid	Useful in food preservation, biodegradable plastics, and green solvents	Wu et al., 2023

Altogether, current finding underscores the high potential of brewery wastewater as a platform for generating novel lipid and protein sources (Dias et al., 2022), while simultaneously reducing environmental impact and promoting circular bioeconomy principles.

Beer wastewater application in agriculture

The global pursuit of sustainable water resources in agriculture has accelerated interest in unconventional alternatives such as treated industrial effluents, with BWW emerging as a promising candidate due to its high organic load and nutrient-rich profile. Given its composition, often containing nitrogen, phosphorus, potassium, and trace micronutrients, BWW can support plant growth while simultaneously addressing water scarcity and waste disposal challenges. Notably, studies have shown that the implementation of crop rotation to mitigate micronutrient depletion did not lead to significant differences in plant growth, indicating that short-term cultivation using BWW may not pose an immediate risk of micronutrient deficiency in soils (Gorfie et al., 2022). Experimental trials (Salian et al., 2018) using a 50:50 mix of BWW and freshwater over five-day germination periods have demonstrated positive effects on seed germination and early growth in crops such as chickpea, corn, and pigeon pea. Similarly, other research supports the use of treated wastewater in agriculture, showing no significant adverse effects on crop yield or quality, with crops like sweet corn and silage maize identified as especially suitable for such irrigation practices (Mok et al., 2014). However, long-term application must be approached with caution, as the accumulation of salts and other residual compounds in soil could impair its structure and fertility, making regular soil monitoring essential to avoid salinity-related issues (Muyen et al., 2011). In the broader context, mounting concerns over water scarcity (Leijnse et al., 2024), declining soil health, and increasing environmental pollution (Rathod et al., 2024) exacerbated by rapid urban expansion, underscore the urgency of integrated wastewater management strategies that align with environmental and agricultural sustainability goals. Traditional wastewater treatment models, focused solely on pollutant removal, must evolve into resource recovery-oriented systems that facilitate the reclamation of nutrients for food production and ecosystem restoration. In this regard, BWW stands out as a readily available and underutilized resource for agricultural irrigation and nutrient cycling,

particularly for recovering plant-essential macronutrients such as nitrogen and phosphorus (Carnevale Miino et al., 2025). Moreover, to reinforce economic circularity and sustainable brewery practices, recent studies (Voss et al., 2024; Cichocki et al., 2025) propose innovative nutrient recovery models from BWW, enabling the production of organic liquid fertilizers suitable for hydroponic systems. This approach not only addresses environmental and economic pressures on the brewing industry but also aligns with growing consumer demand for eco-friendly, traceable agricultural inputs.

CONCLUSIONS

BWW traditionally is viewed as an environmental liability, is now emerging as a valuable resource in the context of sustainable development and circular economy principles. This manuscript highlights the multifaceted potential of BWW, emphasizing its application across water reuse, renewable energy generation, biotechnology, and agriculture. In biotechnology, BWW could serve as a low-cost substrate for cultivating oleaginous microorganisms and producing single-cell protein, lipids, and carotenoids biomolecules with growing demand in the food, feed, and nutraceutical sectors. In agriculture, short-term studies have shown that BWW, when appropriately treated, supports crop development without compromising soil health or plant quality, provided that salinity and contaminant levels are carefully monitored. Overall, the valorisation of brewery wastewater presents an integrative approach to environmental management, resource efficiency, and industrial innovation. Realizing its full potential, however, requires coordinated efforts involving technological advancement, policy support, and cross-sector collaboration. By shifting from linear waste disposal models to resource recovery systems, breweries can not only reduce their ecological footprint but also contribute to broader global goals related to food security, water conservation, and sustainable production systems.

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REFORMULATION OF AN ORGAN-BASED MEAT PRODUCT WITH PUMPKIN POWDER: A STRATEGY FOR FIBER ENRICHMENT

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Abstract

The development and manufacturing of food products, across all sectors of the industry, that align with contemporary dietary habits and meet consumers' nutritional requirements have become a permanent interest of the food industry. This study aims to combine elements of sustainability, superior valorization and the concept of functional products by optimizing the valorization of poultry organs through the reformulation of conventional Leberwurst. To achieve this, pumpkin powder (Cucurbita maxima) was incorporated at concentrations of 0.1%, 3%, and 5% into the product made with liver and chicken thigh, to enrich the product with dietary fibers - an essential nutritional element absent in conventional meat products - while preserving its sensory acceptability and improving its functional and nutritional profile. After production, the batches were subjected to physicochemical evaluations, and characterized in terms of pH, color, texture, and sensory perception. The addition of pumpkin powder results in an improvement in the fiber content, with a good sensory acceptability for all the formulations. This research supports the development of functional meat products with improved nutritional quality and sustainability by valorizing underused ingredients.

Key words*: fiber-rich food, meat product innovation, pumpkin flour.*

INTRODUCTION

In a brief historical overview of the concept of functional foods, Alongi & Anese (2021) mention the first appearance of this term in 1984, in Japan, where the government defined a new category of products, Food for Specific Health Uses, as those foods that contain an ingredient involved in specific functions and physiological effects on human health.

In recent years, numerous authors have highlighted the trend in food industry towards the development of functional products formulated with various additions of bioactive ingredients, such as flax seeds and tomato powder (Ghafouri-Oskuei et al., 2020), quinoa (Fernández-López et al., 2020), chia flour (Fernández-López et al., 2019), sea buckthorn oil (Anchidin et al., 2023), citrus by-products (Nieto & Peñalver, 2021). Moreover, these functional additives can serve a dual role, enriching the nutritional and energy intake and ability to provide a certain degree of protection against the development of chronic diseases (Ampofo & Abbey, 2021; Aziz et al., 2021).

From a scientific perspective, meat is defined as skeletal muscle and the associated tissues, such as adipose, epithelial, connective, and nervous tissues, with the main component being muscle tissue, derived from mammals, birds, reptiles, amphibians, and aquatic species, and harvested for human consumption. Edible organs are also considered meat, consisting of organs and non-skeletal muscle tissues (Boler & Woerner, 2017; Seman et al., 2018). The liver is included in the category of edible organs, which, along with meat, is an important source of micronutrients such as vitamin A and essential minerals including iron, zinc, copper, and manganese (Choe et al., 2019; Boișteanu et al., 2024).

Considering the growth observed in the poultry slaughtering sector - driven by increased consumption associated with population and income growth - there is a growing interest in identifying solutions for the superior valorization of all by-products generated during this process (Gucianu et al., 2024). One of the most effective approaches to utilizing liver is represented by the production of pâté or sausages, well-appreciated varieties in many European countries, such as Denmark, France, Germany, and Spain (Estévez et al., 2005;

Cairncross, 2020). Traditionally, meat products are valued for their high protein content, known as "high-quality" proteins due to the substantial amounts of essential amino acids and elevated bioavailability (Bohrer, 2017; Anchidin et al., 2024). However, these products are naturally low in dietary fibre, a key component for maintaining digestive health (Boișteanu et al., 2023; Haque et al., 2025).

Among the plant fiber sources used in the reformulation of meat products, pumpkin (*Cucurbita maxima*) represents a promising option due to its high dietary fibers content, antioxidants, and bioactive compounds. Pumpkin pulp not only improves the nutritional value of the product but can also contribute to optimizing its texture and stability during thermal treatment and storage (Batool et al., 2021). Several studies in the literature have demonstrated the positive effects of adding pumpkin powder on the functional and technological properties of various food products, such as meatballs (Öztürk & Turhan, 2020), ham (Galenko et al., 2021), sausages (Choi et al., 2012; Hleap-Zapata et al., 2020), and burgers (Hartmann et al., 2020; de Melo et al., 2024). The addition of pumpkin flour to the manufactured product falls into the category of food ingredients rather than food additives, enhancing consumer acceptability, particularly in the context of consumer perception that “synthetic equals dangerous” (Zugravu et al., 2017).

Although a general uniformity in eating behavior has been observed at the national level, with minimal differences between urban and rural areas in terms of meat consumption, meat continues to represent an important component of the population's diet. However, the average consumption remains below nutritional recommendations, especially among women (Pogurschi et al., 2018).

In recent years, consumer interest in poultry meat has increased for various reasons such as accessibility, ease of culinary processing, high protein content, lack of religious restrictions, and the lower environmental impact of the poultry industry compared to the beef sector (Tudorache et al., 2022; Popa et al., 2022; Grigore et al., 2023).

In this context, the development of innovative products, such as chicken sausages enriched pumpkin flour, can contribute to the diversification of the meat product varieties and improve their nutritional profile (through the additional intake of dietary fibers, natural antioxidants, and bioactive compounds derived from plant-based raw materials).

This study aims to investigate the impact of adding pumpkin flour on the physicochemical and sensory characteristics of chicken liver sausages, with the goal of formulating a fibre-enriched meat product with superior nutritional benefits. The reformulation tested the incorporation of three levels of pumpkin flour (1%, 3%, 5%) and a control batch without the additive, monitoring changes in chemical composition, physicochemical properties, and consumer acceptability.

MATERIALS AND METHODS

The study involved two stages: the production stage of the formulations and the qualitative analysis stage of the finished product, carried out in the production departments and laboratories of the "Ion Ionescu de la Brad" Iași University of Life Sciences.

The production process was carried out according to the procedures described in previous studies (Ciobanu et al., 2023a; Manoliu et al., 2023; Flocea et al., 2024), with some modifications specific to the type of emulsified product. The necessary raw and auxiliary materials were purchased from local suppliers, and the pumpkin flour was purchased from Bio Market (Bio Intercom SRL, Cluj-Napoca).

The quantities used for producing the four sausage variants are presented in Table 1. The percentages of pumpkin flour added (1%, 3%, and 5%) were reported to the total amount of raw material used.

The main production stages involved the coarse grinding of meat (using a Grinder WP - 105, equipped with a 3 mm sieve), followed by fine grinding in a Cutter Titane V 45 L, emulsification, addition of dry ingredients, stuffing into natural casings, and thermal treatment, performed according to the protocol outlined in Table 2. After production, the products were cooled and stored at refrigeration temperature (0-3°C) until the subsequent analysis stages.

Table 1. Formulations of liver sausages (chicken Leberwurst) with different concentrations of pumpkin flour

Ingredients / Sample code	Chicken liver (kg)	Chicken deboned thigh (kg)	Pumpkin flour (g)	Salt (g)	Onion powder (g)	Black pepper (g)	Sweet paprika (g)
Control, 0%	1.2	0.8	0	30	20	4	2
CLS, 1%			20				
CLS, 3%			60				
CLS, 5%			100				

Table 2. The heat treatment scheme for the formulations of chicken Leberwurst

Heat treatment stage	Time	Temperature inside the cell	Temperature in the thermal centre	Humidity
	minutes	°C	°C	%
Air drying	30	60	52	30
Smoking	20	70	65	30
Boiling	30	78	72	99
Hot air drying	5	80	70	20

The final products were subjected to physicochemical and sensory evaluations at the Meat and Meat Products Technology Laboratory and Sensory Analysis Laboratory.

The instrumental colour parameters (L*, a* and b*) were assessed using Chroma Meter CR-410 instrument (Konica Minolta Inc., Japan), with the following characteristics: D65 light source, with a 2° observation angle and a 50 mm diameter measurement area. The color attributes chroma (C*), hue angle (H*) and total color difference (ΔE) were calculated using equations (1), (2), and (3), as described in a previous study (Ciobanu et al., 2024a).

$$H(*) = tan^{-1}(\frac{b^*}{a^*}) \quad (1)$$

$$Chroma\ (C^*) = \sqrt{(a*)^2 + (b*)^2} \quad (2)$$

$$\Delta E = \sqrt{(L*s - L*con)^2 + (a*s - a*con)^2 + (b*s - b*con)^2} \quad (3)$$

The acidity of the products, expressed as pH value, was measured using a direct insertion pH meter (HI98163, Hanna Instruments Romania).

For the determination of textural parameters, a Lloyd Instruments TA1Plus texture analyzer (AMETEK, UK) equipped with a 500N load cell was used. Cutting tests were applied using the Warner-Bratzler knife, and compression tests were conducted using a cylindrical probe. The obtained results describe parameters such as shear force (N), work of shear (mJ), and the textural profile analysis attributes: hardness (maximum force during the first compression); cohesiveness, elasticity, gumminess, chewiness, and adhesiveness.

Sample preparation for the Warner-Bratzler shear test involved longitudinally slicing the sausages into 30 × 15 mm strips, both with and without membrane, while for the compression test, sections of approximately 3 cm height were formed.

The raw chemical composition was determined using the NIR FoodCheck analyzer (Bruins Instruments, OmegAnalyzer), and the total fiber content was determined by the AOAC 991.43 method (AOAC, 2000).

Consumer behavior towards a new product does not always correspond to their real needs, but is based on various attitudes and perceptions influenced by: psychological factors (such as product familiarity and openness to innovation) (Ciobotaru et al., 2024), which affect the acceptance decision; and environmental factors that include economic, social, cultural, or consumption context criteria (Ciobanu et al., 2023b).

The sensory analysis was conducted using the method described by Boișteanu et al. (2025), applying the hedonic ranking test for the main sensory attributes (surface appearance, section appearance, color, hardness, adhesiveness, flavor, taste, overall acceptability) to a group of 30 semi-trained panelists. The panelists were asked to rate each sensory attribute on a nine-point scale (1 - dislike very much and 9 - like very much).

The results for physicochemical and sensory quality evaluation of the four sausage samples were expressed as mean values (± standard deviation) and the experimental addes samples were compared with the control using analysis of variance (ANOVA) followed by Tukey's test at 5% significance level ($p < 0.05$), using IBM SPSS Statistics v.21.

RESULTS AND DISCUSSIONS

The results for chemical composition obtained for the chicken Leberwurst formulations are shown in Table 3. All compositional parameters (moisture, fat, protein, ash, fibre and carbohydrate) of chicken Leberwurst sausages were influenced by the addition of pumpkin flour, and the moisture and protein contents decreased as the amount of pumpkin flour increased, while fat, fibre, ash, and carbohydrate contents increased.

Moisture content in meat products is a parameter that influences shelf life and sensory characteristics (particularly texture and juiciness). The incorporation of pumpkin flour led to a progressive decrease in moisture content across all samples, from 72.26% in the control to 70.28% in the 5% supplemented sample. This trend is likely due to the low inherent moisture of pumpkin flour (10.96%, compared to 92.24% in fresh pumpkin) and its high water-binding capacity (Saeleaw & Schleining, 2011), which reduces free water in the meat matrix. Such findings align with previous studies where the addition of pumpkin flour to meat products resulted in decreased moisture levels, enhancing shelf stability (Öztürk & Turhan, 2020; de Melo et al., 2024). Öztürk & Turhan (2020) found that the inclusion of pumpkin (*Cucurbita pepo* L.) seed kernel flour into beef meatballs led to reduces moisture from 47.34% in control sample to 44.74% in the 12% added sample.

Interestingly, a slight but statistically significant ($p < 0.05$) reduction in protein content was observed with the increasing pumpkin flour levels (from 20.42% in the control to 20.06% at 5%). Hleap-Zapata et al. (2020) also reported this effect of reduced protein content in Frankfurt-type sausages after the addition of pumpkin flour (*Cucurbita moschata* Duch.), from a mean value of 34.55% (control sample) to 29.88% (in the 30% replacement of wheat flour with pumpkin flour). This can be explained by a dilution effect, as plant-based ingredients typically have lower protein levels than lean chicken meat. Contrarily, other studies by Öztürk & Turhan (2020) and de Melo et al. (2024) showed an increase in protein content when adding pumpkin seed flour.

The fat content showed a progressive and statistically significant ($p < 0.05$) increase in the supplemented chicken Leberwurst (from 4.94% in the control to 5.74% at 5% addition). This trend could be attributed to the lipid contribution of the pumpkin flour itself, or more likely, a concentration effect due to moisture reduction. A similar increase in fat concentration following pumpkin addition was noted by Zargar et al. (2014) in pumpkin-enriched chicken sausages.

A clear increasing trend was observed in carbohydrate, fibre and ash contents, reflecting the high content of non-digestible polysaccharides naturally present in pumpkin flour. The carbohydrate content increased from 0.96% in the control to 1.94% in the 5% added chicken Leberwurst, while dietary fibre rose from 0% (in the plain meat sausages) to a maximun result of 1.03%, with statistically significant differences between all samples ($p < 0.05$). Similar results were also reported by de Melo et al. (2024), who found an increase in total fibre content from 0% to 0.88% at a 5% level of pumpkin seed flour incorporated in beef burgers. These results confirm the functional enrichment achieved through pumpkin flour addition, which enhances the nutritional profile by introducing fermentable fibre known for its beneficial effects on gut health (Haque et al., 2025).

In terms of ash content, a marker of mineral content, the significant increase with pumpkin flour addition (from 1.42% to 1.98%) can be due to the natural mineral load of pumpkin pulp, which contains potassium, calcium, and magnesium (El Khatib & Muhieddine, 2020). This is consistent with other studies that also reported increased ash values in pumpkin-fortified meat products (Verma et al., 2015; Hartmann et al., 2020; Öztürk & Turhan, 2020).

Table 3. Analysis of chemical composition of control and pumpkin flour supplemented chicken Leberwurst

Analyzed parameter	Sample			
	Control, 0%	CLS, 1%	CLS, 3%	CLS, 5%
Humidity (%)	72.26 ± 0.18 ^{a}	71.32 ± 0.28 ^{b}	71.08 ± 0.16 ^{b}	70.28 ± 0.13 ^{c}
Dry Matter (%)	27.74 ± 0.18 ^{c}	28.68 ± 0.28 ^{b}	28.92 ± 0.16 ^{b}	29.72 ± 0.13 ^{a}
Fat (%)	4.94 ± 0.11 ^{c}	5.08 ± 0.04 ^{bc}	5.16 ± 0.11 ^{b}	5.74 ± 0.15 ^{a}
Protein (%)	20.42 ± 0.18 ^{a}	20.32 ± 0.13 ^{ab}	20.18 ± 0.04 ^{bc}	20.06 ± 0.05 ^{c}
Colagen*	19.56 ± 0.09 ^{a}	19.52 ± 0.04 ^{a}	19.48 ± 0.08 ^{a}	19.42 ± 0.13 ^{a}
Carbohydrate (%)	0.96 ± 0.05 ^{b}	1.56 ± 0.31 ^{a}	1.72 ± 0.24 ^{a}	1.94 ± 0.17 ^{a}
Fiber (%)	0.00 ± 0.00 ^{c}	0.71 ± 0.14 ^{b}	0.74 ± 0.12 ^{b}	1.03 ± 0.10 ^{a}
Ash (%)	1.42 ± 0.19 ^{c}	1.72 ± 0.08 ^{b}	1.86 ± 0.11 ^{ab}	1.98 ± 0.13 ^{a}

Values are given as means ± Standard deviation from five repeated determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test. *Colagen content is expressed as % from the total protein content.

Table 4 shows the mean results registered for pH and color parameters (L*, a*, b*, C*, h*, and ΔE) for the sausage samples, and also for the pumpkin flour. These phisical parameters are determinant of consumer acceptance, serving as key indicators of product quality.

The pH values remained relatively stable across all samples, ranging from 6.68 to 6.74, with no significant differences observed ($p > 0.05$), although it recorded a decrease with the increase in the level of pumpkin flour. This stability suggests that the addition of pumpkin flour, even at the highest concentrations, does not significantly modify the acidity or alkalinity of the product, maintaining the physicochemical environment essential for product stability and shelf life.

The general definition of the main color parameters is: L* = 0 (black); L* = 100 (white); negative a* = green color; positive a* = red color; negative b* = blue color; positive b* = yellow (Anchidin et al., 2023).

The lightness (L* values) and yellow/blue coordinate (b* value) of the chicken Leberwurst exhibited no significant changes ($p > 0.05$) with the addition of pumpkin flour, maintaining values of 40.89-41.55, respectively 10.80-11.13. This consistency indicates that pumpkin flour has a balanced color ant its incorporation does not affect the color characteristics of the meat product, preserving its visual brightness.

A significant decrease in a* values ($p < 0.05$) was observed with increasing levels of pumpkin flour, dropping from 24.70 in the control chicken Leberwurst to 22.93 in the 5% pumpkin flour supplemented formulation. This reduction in red color intensity could be attributed to the dilution of meat pigments, caused by the natural color of the flour, that registered a negative a* value (-0.73), due to pigments in pumpkin flour that influences the overall color balance of the meat product.

Chroma values (C*), representing color saturation, showed a decreasing tendency with higher pumpkin flour concentrations, from 27.03 in the control to 25.49 in the 5% sample.

While hue angle (h*) recorded a reverse trend compared to chroma, increasing significantly with higher levels of pumpkin flour, from 23.97 in the control to 25.90 in the 5% sample ($p < 0.05$). This decline in saturation indicates a slight reduction in color intensity, and an increased hue angle suggests a shift in color perception, potentially moving towards a more yellowish hue, which aligns with the natural color properties of pumpkin.

The ΔE values, which quantify the overall color difference between the experimental samples and the control, ranged from 0.91 to 1.97 across the samples. According to Wibowo et al. (2015), ΔE values between 0.5 and 1.5 are considered slightly visible, while values between 1.5 and 3.0 are noticeable. Therefore, the color changes observed with pumpkin flour addition are perceptible but remain within acceptable limits, the 3% and 5% levels producing a more evident color difference compared to the control sample. Similar results have also been reported by other authors who studied the potential of incorporating pumpkin flour into various meat products (e.g., beef burgers, beef meatballs, beef patties), demonstrating a slight increase in brightness and color intensity towards yellow (b* value) and a decrease in the a* value, through the dilution of the meat's red pigment (Ammar et al., 2014; Serdaroğlu et al., 2018; Öztürk & Turhan, 2020; de Melo et al., 2024).

Table 4. pH and colorimetric parameters of pumpkin flour and chicken Leberwurst (control and supplemented formulations)

Analyzed parameter	Pumpkin flour	Leberwurst sample			
		Control, 0%	CLS, 1%	CLS, 3%	CLS, 5%
pH	-	6.74 ± 0.08 [a]	6.71 ± 0.08 [a]	6.69 ± 0.07 [a]	6.68 ± 0.06 [a]
L*(D65)	70.49 ± 0.56	41.03 ± 0.73 [a]	40.89 ± 0.77 [a]	41.08 ± 0.59 [a]	41.55 ± 0.90 [a]
a*(D65)	-0.73 ± 0.20	24.70 ± 0.60 [a]	24.19 ± 0.29 [ab]	23.50 ± 0.73 [bc]	22.93 ± 0.27 [c]
b*(D65)	25.63 ± 0.18	10.98 ± 0.28 [a]	10.80 ± 0.11 [a]	10.89 ± 0.29 [a]	11.13 ± 0.29 [a]
C*(Chroma-Saturation index)	25.64 ± 0.17	27.03 ± 0.64 [a]	26.49 ± 0.31 [ab]	25.90 ± 0.75 [bc]	25.49 ± 0.13 [c]
h* (Hue angle)	91.64 ± 0.45	23.97 ± 0.31 [b]	24.07 ± 0.10 [b]	24.88 ± 0.59 [b]	25.90 ± 0.83 [a]
ΔE	-	-	0.91 ± 0.46	1.76 ± 0.73	1.97 ± 0.70

Values are given as means ± Standard deviation from five repeated determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

Textural attributes are important elements in the sensory perception and consumer acceptance of meat products, influencing attributes such as mouthfeel, chewiness, and slicing behavior. In this study, the effect of inclusion of pumpkin flour in varying concentrations (1%, 3%, and 5%) was measured instrumentally for shear test and textural profile parameters, on samples with and without membrane. As can be seen in Table 5, the presence of the membrane prevented the identification of significant differences between samples regarding TPA parameters, while the shear force (N/cm) of sausages with membrane displayed a gradual and significant reduction ($p < 0.05$) with increasing pumpkin flour addition - from 4.65 N/cm in the control sample to 3.14 N/cm in the 5% sample.

Despite the trend in shear resistance, the textural parameters hardness, cohesiveness, elasticity, gumminess, and chewiness values did not vary significantly ($p > 0.05$) between treatments, though a slight numerical decline was observed in most parameters with increased pumpkin flour.

The reverse trend was observed in samples without membrane, where shear force increased with increasing pumpkin flour content - from 1.18 N/cm (control) to 1.58 N/cm (5%), indicating a firmer internal meat matrix after the addition of pumpkin flour. The work of shear (mJ) also increased significantly ($p < 0.05$), suggesting that flour addition contributed to a stronger internal structure.

The hardness results followed a similar pattern, increasing from 3.86 N to 5.23 N, at the highest level of addition the sample being significantly firmer ($p < 0.05$). This may reflect the water-holding and swelling behavior of pumpkin flour within the protein gel, leading to increased matrix density. In terms of cohesiveness, elasticity and chewiness, the samples remained statistically similar, while gumminess has significantly decreased (from 1.21 N to 0.78 N), suggesting that the addition may have disrupted the viscoelastic matrix, facilitating easier breakdown during mastication.

The differences in behavior of the samples with and without membrane, at the instrumental measurement of texture parameters, can be attributed to the structural role of the natural casing and its interaction with the internal meat matrix. In the control samples (0% pumpkin flour), the natural pig membrane was elastic, and did not adhere tightly to the meat paste, resulting in higher shear force values due primarily to the resistance of the membrane itself. Moreover, the control chicken Leberwurst had a softer, light consistency, offering minimal internal resistance, which concentrated the cutting effort on penetrating the membrane.

In contrast, the addition of pumpkin flour enhanced the water-binding capacity and firmness of the meat matrix, promoting a more compact and cohesive internal structure. As a result, the membrane became better adhered to the surface of the meat paste and more integrated into the product, likely due to reduced moisture exudation and improved surface contact. This led to lower shear force values, as the cutting blade could move through a more continuous matrix with less membrane deformation.

On the other hand, after removing the membrane, the shear force increased with the level of pumpkin flour, reflecting the true internal firmness of the product. Without the natural membrane's influence, the cutting resistance was directly related to the structural integrity of the meat-pumpkin flour matrix.

Here, the progressive increase in shear force values and hardness demonstrates that pumpkin flour contributed to the densification and strengthening of the emulsion, resulting in a firmer core that required more energy to shear.

Table 5. Warner Bratzler shear forces and textural parameters of the control and pumpkin flour supplemented chicken Leberwurst

Analyzed parameter	Sample			
	Control, 0%	CLS, 1%	CLS, 3%	CLS, 5%
with membrane				
Shear force (N/cm^2)	4.65 ± 1.29 ^{a}	4.03 ± 0.64 ^{ab}	3.54 ± 0.25 ^{ab}	3.14 ± 0.35 ^{b}
Work of shear (mJ)	326.26 ± 9.35 ^{a}	276.68 ± 7.51 ^{b}	246.28 ± 5.21 ^{c}	197.99 ± 8.86 ^{d}
Hardness (N)	14.52 ± 3.81 ^{a}	12.63 ± 1.83 ^{a}	10.69 ± 0.78 ^{a}	10.84 ± 2.09 ^{a}
Cohesiveness (N)	0.33 ± 0.17 ^{a}	0.32 ± 0.03 ^{a}	0.27 ± 0.19 ^{a}	0.11 ± 0.03 ^{a}
Elasticity	0.36 ± 0.09 ^{a}	0.30 ± 0.05 ^{a}	0.22 ± 0.04 ^{a}	0.27 ± 0.17 ^{a}
Gumminess (N)	2.78 ± 0.69 ^{a}	2.56 ± 0.17 ^{a}	2.18 ± 0.22 ^{a}	2.09 ± 0.53 ^{a}
Chewiness (N)	1.04 ± 0.47 ^{a}	0.77 ± 0.18 ^{a}	0.49 ± 0.15 ^{a}	0.61 ± 0.52 ^{a}
Adhesiveness	-0.55 ± 0.01 ^{a}	-0.58 ± 0.03 ^{a}	-0.46 ± 0.01 ^{a}	-0.52 ± 0.15 ^{a}
without membrane				
Shear force (N/cm^2)	1.18 ± 0.23 ^{b}	1.27 ± 0.23 ^{ab}	1.42 ± 0.22 ^{ab}	1.58 ± 0.07 ^{a}
Work of shear (mJ)	87.99 ± 6.77 ^{c}	103.52 ± 8.51 ^{b}	103.89 ± 2.69 ^{b}	116.50 ± 8.60 ^{a}
Hardness (N)	3.86 ± 0.77 ^{b}	4.10 ± 0.86 ^{ab}	4.33 ± 0.72 ^{ab}	5.23 ± 0.17 ^{a}
Cohesiveness (N)	0.22 ± 0.13 ^{a}	0.13 ± 0.03 ^{a}	0.15 ± 0.02 ^{a}	0.16 ± 0.05 ^{a}
Elasticity	0.07 ± 0.01 ^{b}	0.07 ± 0.01 ^{b}	0.09 ± 0.01 ^{a}	0.10 ± 0.01 ^{a}
Gumminess (N)	1.21 ± 0.14 ^{a}	1.06 ± 0.29 ^{ab}	0.89 ± 0.17 ^{ab}	0.78 ± 0.16 ^{b}
Chewiness (N)	0.08 ± 0.02 ^{a}	0.07 ± 0.02 ^{a}	0.09 ± 0.01 ^{a}	0.07 ± 0.01 ^{a}
Adhesiveness	-0.97 ± 0.01 ^{a}	-0.72 ± 0.34 ^{a}	-0.71 ± 0.36 ^{a}	-0.47 ± 0.29 ^{a}

Values are given as means ± Standard deviation from five repeated determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

Table 6 shows the results received in the hedonic consumer test for chicken Leberwurst samples formulated with different levels of pumpkin flour (1%, 3%, and 5%) compared to the control sample. The evaluated attributes included appearance, section appearance, color, hardness, adhesiveness, flavor, taste, and overall acceptability. Given the numerous reports regarding the potential risk of synthetic additives, increasing emphasis is being placed on ”clean-label” products that replace synthetic additives with natural substitutes, offering healthier alternatives without significantly impacting sensory characteristics (Ciobanu, 2024b).

The results reveal that consumer perception on surface appearance, section appearance, and color remained relatively stable between the control and the 1% and 3% samples, with mean values consistently above 7. However, a notable decline was observed for the 5% sample, particularly for section appearance (6.50 ± 1.22) and color (6.43 ± 1.76), suggesting that higher levels of pumpkin flour may have negatively influenced the visual appeal of the product. This sensory perception aligns with the visible decrease in a* and C* values previously discussed in the instrumental color analysis. Textural attributes such as hardness and adhesiveness were positively perceived in the 1% and 3% supplemented samples, with adhesiveness showing a marked improvement from 3.03 ± 1.97 in the control to 6.80 ± 1.65 in the 1% sample. However, both parameters declined at the 5% inclusion level, suggesting that consumer-perceived texture was optimal at moderate enrichment. Considering that the evaluation scale was based on the degree of acceptability, the textural attributes of the control sample received lower scores from the panelists due to its soft consistency and high adhesiveness.

Regarding flavor and taste, all enriched samples show high hedonic scores, and the CLS3% sample scoring the highest acceptability regarding both flavor (8.20 ± 0.76) and taste (8.10 ± 0.88). The CLS5% sample maintained similar flavor and taste scores to that of the control, suggesting that, despite visual and textural changes, the sensory perception of flavor remained constant and palatable.

All samples were positively rated for overall acceptability, with the highest score (7.33 ±

0.80) going to the 3% sample, followed by 1% and 5% additions, and the control. This indicates that moderate amounts of pumpkin flour (1-3%) can improve the sensory profile of the product, while excess amount (5%) may interfere with certain sensory attributes (especially visual and textural features).

Table 6. Sensory attributes of control and pumpkin flour supplemented chicken Leberwurst

Evaluated attribute	Sample			
	Control, 0% ±	CLS, 1%	CLS, 3%	CLS, 5%
Appearance	7.33 ± 1.12 [a]	7.57 ± 1.33 [a]	7.40 ± 0.93 [a]	6.73 ± 1.17 [b]
Section appearance	7.20 ± 0.92 [ab]	7.33 ± 1.21 [a]	7.27 ± 0.91 [a]	6.50 ± 1.22 [b]
Color	7.53 ± 0.94 [a]	7.67 ± 1.03 [a]	7.60 ± 1.19 [a]	6.43 ± 1.76 [b]
Hardness	5.13 ± 1.31 [b]	6.47 ± 1.22 [a]	6.63 ± 1.13 [a]	5.50 ± 1.22 [b]
Adhesiveness	3.03 ± 1.97 [c]	6.80 ± 1.65 [a]	6.43 ± 1.74 [a]	4.77 ± 0.94 [b]
Flavor	7.73 ± 0.94 [a]	8.03 ± 1.03 [a]	8.20 ± 0.76 [a]	7.73 ± 1.01 [a]
Taste	7.90 ± 0.96 [a]	8.13 ± 0.94 [a]	8.10 ± 0.88 [a]	7.80 ± 0.85 [a]
Overall acceptability	7.07 ± 0.78 [a]	7.27 ±0.87 [a]	7.33 ± 0.80 [a]	7.13 ± 0.90 [a]

Values are given as means ± Standard deviation. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

CONCLUSIONS

This study explored the effects of incorporating pumpkin flour into a organ-based chicken sausages (Leberwurst) in order to obtain a reformulated product with functional properties, specifically its enrichment with dietary fiber.

This study demonstrates the feasibility of using pumpkin flour (1%, 3%, and 5%) in the development of chicken Leberwurst with enhanced nutritional value, particularly through significant increases in dietary fibre, carbohydrate, and ash content. Meanwhile, the moisture and protein contents decreased slightly as a result of the additive's composition and moisture-binding properties. These results not only show an improvement in the nutritional profile, but also supports the posibility of developing a functional meat product.

From a technological perspective, pumpkin flour incorporation influenced both textural and color parameters. Color attributes such as green-red coordonate (a*) and saturation (C*) decreased slightly, while the hue angle increased, indicating a subtle but acceptable shift in color, which remained within consumer-acceptable limits based on ΔE values.

In terms of textural modifications, the addition of pumpkin flour resulted in a progressive increase in shear force, work of shear, and hardness, indicating a firmer and more cohesive product. These effects can be attributed to the water-holding and gel-forming abilities of the added flour, which enhanced the structural density of the product. At the same time, gumminess and elasticity were influenced by the addition, but the chewiness and cohesiveness remained stable, ensuring an acceptable sensory texture profile.

Overall, the results confirm that pumpkin flour can be used up to a 5% inclusion level to formulate a clean label, fibre-enriched chicken Leberwurst, without negatively affecting key quality attributes.

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ANALYSIS OF TECHNOLOGICAL, SENSORY AND FOOD SAFETY CHARACTERISTICS OF SEMI-PREPARED PRODUCTS MADE FROM CHICKEN INNER FILET

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Abstract

This paper provides a comparative evaluation of three semi-prepared products made from chicken inner fillet, differentiated by the type of coating applied: classic breadcrumbs, corn flakes, and pumpkin seeds. The study integrates technological, sensory, and food safety perspectives to assess the impact of each variant on thermal behavior, oil absorption, structural stability, and consumer acceptance. Technological parameters such as core temperature during thermal treatment, freezing dynamics, and the performance of critical control points (CCPs) were analyzed in relation to the crust type. Microbiological and chemical analyses confirmed the safety of all products, with results complying with current European regulations. Nutritional profiling revealed the superior functional value of the pumpkin seed variant, while sensory evaluation favored the corn flakes product due to its crispy texture and appealing flavor. These findings support the optimization of technological workflows and the development of safe, high-quality products tailored to the demands and preferences of consumers.

Key words*: critical control points, food safety, functional coatings, inner fillet, nutritional profile, sensory evaluation.*

INTRODUCTION

Meat is a staple food in the human nutritional structure, being considered a concentrated source of proteins with high biological value, essential amino acids, fatty acids, water-soluble vitamins of the B complex, as well as bioavailable minerals such as heme iron or zinc (Stanciu et al., 2015; Ianiţchi et al., 2024). In parallel with this physiological importance, meat consumption reflects a number of socio-economic influences, being sensitive to factors such as urbanization, globalization of supply chains and increasing awareness of healthy eating (Curea et al., 2023; Moise et al., 2024). In this context, poultry meat has recorded a steady positive dynamic in consumer preferences, due to its favorable nutritional profile, technological versatility and relatively affordable cost (Almeida et al., 2015; Costache et al., 2019; Usturoi et al., 2021; Tudorache et al., 2022). According to the data published by the Food and Agriculture Organization of the United Nations (FAO), in 2021 Romania recorded an average annual consumption of 22 kg per capita of poultry meat, expressed as net edible weight, which consolidates this category in second place in the hierarchy of national consumption, after pork (FAO, 2023). This development is correlated with the expansion of the segment of semi-prepared products, in particular poultry meat products, which simultaneously meet the requirements of speed of preparation, long shelf life and food safety (Chugunova et al., 2021; Giucă et al., 2023; Custură et al., 2024). Semi-prepared poultry-based products occupy a central place in the diversification strategy of the food industry, due to their potential to combine attractive sensory characteristics with microbiological safety and high nutritional value (Custură et al., 2019; Gheorghe et al., 2022b; Antoniv & Adamchuk, 2024). These products usually involve the application of a partial heat treatment followed by blast freezing, with a rigorous control of the technological steps, starting with the selection

of the raw material and ending with the stability of the structure during storage (Boriosova et al., 2021). An essential element in the realization of these products is the crispy coating system, which influences not only the organoleptic profile, but also the thermal behavior and shelf-life. Traditional coatings, such as wheat flour and classical breadcrumbs, are commonly used due to their predictable technological behavior and specific texture contribution (Xu et al., 2025). Flour contributes to surface uniformity and interior moisture retention, while breadcrumbs provide a stable crunchy crust (Chaunier et al., 2007). Corn flakes represent a valuable technological solution due to their ability to give the product an expanded texture, characterized by low density and mechanical properties that favour the auditory perception of crunchiness during consumption, an essential aspect in sensory evaluation (Farroni & Buera, 2014). Their structure and texture are directly dependent on the physical states of water and processing conditions, including gelatinization and starch retrogradation, both when used for the crust and when embedded in the structure of the products (Sumithra & Bhattacharya, 2008; Chaunier et al., 2005; Ianiţchi et al., 2023). Corn flakes, due to their expanded structure and low absorption capacity, represent an optimal technological support for the integration of functional ingredients or for creating a crunchy texture, contributing to the versatility of their application in processed food products (Singu et al., 2020). Pumpkin seeds are increasingly emerging as valuable functional ingredients in modern food formulations due to their complex chemical composition and remarkable nutritional density. They are a significant source of good quality plant proteins, essential fatty acids (especially polyunsaturated), dietary fiber and micronutrients such as zinc, magnesium and iron, as well as bioactive compounds with antioxidant and anti-inflammatory roles, such as phytosterols, tocotrienols and polyphenols (Fatima et al., 2025; Dotto & Chacha, 2020). Previously considered as low-value agro-industrial by-products, pumpkin seeds are now recognized for their contribution to the prevention of chronic diseases, while being promoted as sustainable ingredients compatible with food strategies oriented towards functionality and waste reduction (Syed et al., 2019). Their physico-chemical characterization further reveals technologically relevant properties such as low density, controllable moisture and a favorable absorption capacity, directly impacting their behavior in frying or baking processes (Devi, 2018). These qualities recommend them for integration into meat-based food products, especially in value-added semi-finished products, where they can simultaneously perform structural, sensory and nutritional roles (Fatima et al., 2025). The presence of pathogens such as *Salmonella* spp. or *Listeria monocytogenes* is unacceptable in incompletely heat-treated products, as these agents can survive the initial processing and multiply during improper handling or storage (Kataoka et al., 2017; Simpson-Beauchamp et al., 2010). In addition, physico-chemical indicators such as pH and readily hydrolyzable nitrogen can provide essential information on the freshness and degree of spoilage of meat (Surmei et al., 2013). From the perspective of chemical contaminants, plant sources in the composition of coatings can be exposed to the presence of mycotoxins (DON, aflatoxins, ochratoxin A), heavy metals (lead, cadmium) or newly formed compounds such as acrylamide (Ji et al., 2024; Andrade et al., 2018; Castells et al., 2008). The microbiological and physico-chemical quality of semi-finished poultry meat products is proving to be a critical point in the production chain, given their increased susceptibility to bacterial contamination. This reality calls for the establishment of rigorous processing, handling and storage protocols aimed at minimizing consumer health risks (El-Kewaiey, 2012). The application of modern analytical methods for the detection and quantification of pathogens or chemical contaminants becomes imperative to ensure compliance with European food safety standards (Pogurschi et al., 2022). In tandem, studies on consumer perceptions of semi-prepared products and food labeling show a growing concern for traceability, ingredient content and the potential presence of allergens (Grapă et al., 2022). The use of novel ingredients, such as pumpkin seeds, may also require adaptations in consumer

communication strategies (Silva de Lana et al., 2025). At the same time, the meat industry in Romania is in a process of modernization and adaptation to European standards, supported by investments in technologization and diversification of the range of value-added products (Uliu & Vladu, 2021; Custură et al., 2012; Gheorghe et al., 2022a). Fully valorizing native agro-food resources, including the integration of plant co-products in meat products, is a strategic direction to increase competitiveness (Rățu et al., 2023; Nistor et al., 2015).

Therefore, this work aims to comparatively investigate three variants of semi-finished chicken fillet-based products, differentiated by the coating applied, classic breadcrumbs, corn flakes and pumpkin seeds, from the perspective of sensory, technological and food safety characteristics. The study provides an integrative, science-based approach to optimize formulations and validate them against current market expectations and regulatory requirements.

MATERIALS AND METHODS

The products analyzed in this study were produced in an industrial meat-processing plant using frozen chicken tenderloin (commercially known as "inner" or "tenderloin") as raw material. The fillets were gradually brought to the chilled state to avoid thermal shock and to prevent damage to the muscle structure. Each batch was then X-rayed for bone fragments to ensure that the product complied with food safety requirements. Due to its anatomical fragility and small surface area, an immersion marinating process was used, which ensures that the compounds gradually diffuse into the meat without altering its integrity. The marinade used consisted of a saline solution with plant extracts, water stabilizing agents, natural antioxidants and spices, and was applied under controlled refrigeration to allow uniform diffusion and prevent the risk of microbial growth. Björkroth (2005) demonstrated that in marinated products packaged in modified atmosphere, psychrotrophic lactic acid bacterial species such as *Leuconostoc gasicomitatum* can become dominant, thus contributing to organoleptic defects and shortening shelf life. Prior to marinating, the fillets were portioned using specialized Marel equipment calibrated to meet the weight requirements. This equipment allows high precision portioning, compensating for the extra weight generated by the subsequent crust coating. The technological flow continued with the coating stages, common to all three variants analyzed, up to the point of application of the final breadcrumbs. Initially, each portion was passed through a thin layer of white wheat flour to act as a sticking agent, followed by a first pass through egg mixture. This is a semi-liquid technological mixture of whole liquid egg, skimmed milk, salt and stabilizing agents (food starch or functional proteins) to provide adherence between successive layers. After a second pass through the breadcrumbs, which is intended to reinforce the base layer, the product was prepared for the application of the final layer of breading, specific to each recipe. By analogy with the study by Cakmak et al. (2013), in the case of breaded products, coating meat with a successive layer of flour, breadcrumbs and breadcrumbs can benefit from the same functional principles, in the sense that the interaction between meat proteins and the compounds in the applied layers contributes to better adhesion, stability of the composition during heat treatment and improved sensory characteristics of the crust.

At this stage the three technological variants were differentiated:

Variant 1: the use of standard breadcrumbs, with a medium grain size and medium oil absorption, applied uniformly to ensure a homogeneous crust;

Variant 2: involved the use of shredded corn flakes, which, due to their porous structure and high starch content, provided a more aerated crust, but with an increased capacity for oil absorption during frying;

Variant 3: use of coarsely ground pumpkin seeds, which, due to their high unsaturated lipid content, influenced the final texture, generating a crust with increased density and a specific flavor profile.

Considering the specific nature of the ingredients used in the corn flakes and pumpkin seed variants, it was necessary to implement enhanced sanitation protocols at the

level of technological equipment, in accordance with the principles of good manufacturing practice. This was aimed at preventing cross-contamination between batches, particularly in the context of the risk of exposure to allergens or compounds with the potential to induce food intolerances. The products were then placed in an industrial deep fryer, where they were quickly fried for approximately 28 seconds, sufficient to form a crispy outer crust without excessive oil penetration into the protein mass. After frying, the products were transferred to a professional oven, where they were baked at 120°C for 5 minutes in a controlled environment with 80% steam opening. This parameter was chosen to limit excessive evaporation of water, thus preventing the product from drying out and preserving the characteristic juiciness of the fillet inside. Rigorous control of the internal temperature was essential at this stage. The aim was to achieve a minimum temperature of 72°C in the center of the product, which is considered microbiologically critical. Reaching this level allows the effective inactivation of pathogenic bacteria of major interest in the poultry industry, in particular *Salmonella* spp. and *Listeria monocytogenes* (Ehuwa, 2021), agents commonly associated with poultry meat. Although the product is intended for consumption after further thermal preparation in a domestic environment, compliance with this minimum threshold at the industrial stage provides added food safety in the event that the consumer does not complete the thermal process properly. After the heat treatment, the products were placed on racks and transferred to the blast freezing tunnel, where an internal temperature of -18°C was monitored, in line with industry standards for the safety and microbiological stability of frozen products. According to Barbut (2012), in the case of breaded products, this step is essential not only for safety reasons, but also to ensure good adhesion and stability of the coating during handling and further transportation. Rapid freezing to a minimum internal temperature of -18°C, applied immediately after the heat treatment, prevents the formation of large ice crystals, which could damage the internal texture, preserves the crispness of the breadcrumbs and maintains the structural integrity of the finished product according to current industry standards. The products were then individually packaged according to the customer's requirements, labeled accordingly and stored in temperature-controlled refrigerated rooms.

During the technological flow, potential differences were noted between the three types of coatings used, not only in terms of oil absorption and moisture loss, but also in terms of visual appearance, crust texture, flavor and baking behavior. Given observed technological differences, particularly in visual appearance, crust texture, flavor, and baking behavior, a sensory analysis was subsequently carried out to assess how these variations were perceived by consumers. Sensory analysis was carried out under controlled conditions, in a space dedicated to organoleptic testing, without odor or light interference that would influence the perceptions of the participants. The evaluation panel was made up of 30 naive tasters, aged between 18 and 35, selected from among ordinary consumers, without professional training in sensory analysis. The choice of this age range was not accidental, but was based on current consumer realities: this category of population, professionally and socially active, is frequently associated with an alert lifestyle and limited time available for cooking, aspects that determine a high predisposition to the consumption of semi-prepared products, considered a practical and quick alternative for daily meals.

To ensure compliance with food safety standards and to verify the absence of chemical and microbiological hazards, a series of targeted analyses were performed on both crust components and the final semi-prepared products. The analyses were carried out by accredited laboratories, according to international standards (ISO/IEC 17025), and the methods applied were in full accordance with current European legislation, notably Regulation (EU) No. 915/2023 concerning contaminants in foodstuffs and Regulation (EC) No. 2073/2005 on microbiological criteria.

In the case of the corn flakes coating, chemical contaminant analyses focused on compounds relevant to cereal-based ingredients. The main objectives were to determine the presence and

concentrations of the following mycotoxins: Aflatoxins B1, B2, G1, G2 and total, Fumonisins B1 and B2, Zearalenone, as well as chlorate and perchlorate residues. The applied methods included VICAM AflaTest WB (Instruction Manual # GN-MC9536-0 Rev. B, 2008) for aflatoxins and method MP.2360:R1.2023 for fumonisins and zearalenone. Chlorate and perchlorate were analyzed according to the standard method H-MA-M 02-155, using LC-MS/MS technology (method version: 2023-05).

Considering the oleaginous nature of pumpkin seeds (Leichtweis et al., 2025), which are known to be susceptible to ochratoxin contamination during storage, specific analyses were conducted to detect this mycotoxin. The determination was performed using method MP.2360:R2.2024, adapted to high-lipid matrices.

As for the finished product, the breaded and frozen chicken inner fillet microbiological safety was assessed through determinations for reference pathogens and hygiene indicators. The presence of *Salmonella* spp. was specifically tested in 25 g of product, using the reference method SR EN ISO 6579-1:2017, which is harmonized with EU legislation (Regulation (EC) No. 2073/2005). The analysis was performed on five independent subsamples. Additionally, determinations were conducted for *Escherichia coli*, coagulase-positive Staphylococci, yeasts, molds, and the total viable count, in line with microbiological performance standards.

In the case of the breadcrumb-based variant, the acrylamide content of the classic breadcrumb coating was also determined, due to the known risk of its formation during high-temperature baking. The method applied was MSZ EN 16618:2015, which complies with EU recommendations on process contaminants.

RESULTS AND DISCUSSIONS

The technological differences between the three products analyzed, although subtle in the overall manufacturing flow, become evident at the coating stage and manifest themselves in complex ways in the thermal behavior, sensory characteristics and structural stability of the finished product. Double layering, carried out by applying flour, egg-based breadcrumbs and then a crispy coating (classic breadcrumbs, corn flakes or pumpkin seeds), leads to relevant variations in oil absorption, moisture loss, texture and final appearance.

For products coated with classic breadcrumbs, oil absorption during heat treatment (28 seconds in the oil bath) was in the moderate range, due to the relatively compact structure of the granules and their ability to form a uniform barrier on the surface. This characteristic contributed to a crispy, well-browned crust with a regular appearance and a golden-brown color typical of traditional bread products.

Moisture losses during baking were observed empirically and estimated to range between 4-8%, while still maintaining the juiciness of the interior, a key factor in reaching the core temperature of ≥72°C, maintaining the juiciness of the interior, which is essential for achieving the core temperature of ≥72°C, set as the critical limit under Critical Control Point 1 (CCP1 - heat treatment). Therefore, in the case of breaded products, the heat treatment carried out at industrial level and the confirmation of reaching the target core temperature is not only a technological requirement but an essential food safety measure, fulfilling all the conditions of a Critical Control Point (CCP), according to HACCP principles, as also argued by Molins (2001). The texture was homogeneous and the flavor profile discrete, with no dominant notes interfering with the natural flavor of the meat.

In contrast, products coated with corn flakes exhibited a distinctive behavior. Although the aerated and porous structure of the corn flakes facilitated a visibly higher surface oil uptake during frying, the final fat content was the lowest among all three samples (8.5 g/100 g, Table 2). This seemingly contradictory result can be explained by the inherently low lipid content of corn flakes and the brief duration of frying (28 seconds), which limits oil penetration beyond the surface layer. Moreover, the subsequent baking phase, performed at 120°C for 5 minutes with 80% steam, according to the product's technical processing specification, caused partial evaporation of surface oil., contributing to the lower final fat content. Moisture losses were observed during baking for this variant were

estimated at approximately 7-8%, is attributed to the irregular crust geometry and open structure, that allowed greater steam release. From the PCC1 perspective, this variant required careful monitoring of the internal temperature distribution, given the uneven thermal dispersion associated with the aerated structure of the outer layer.

On the other hand, the products coated with pumpkin seeds showed reduced oil absorption during frying. However, despite the lower frying absorption, this variant recorded the highest overall fat content (11 g/100 g, Table 2), which is attributed to the naturally high lipid concentration in pumpkin seeds themselves. During baking, this variant showed greater moisture retention, but also a denser and less expanded crust. The visual appearance was darker, with a specific greenish-brown color, which led to a less familiar visual perception for the tasters. Aromatically, the coating introduced intense oleaginous and vegetal notes, which slightly dominated the taste of the preparation, thus influencing the sensory evaluation. Due to the dense texture and more inert thermal behavior, a rigorous monitoring of the temperature value in the center of the product (PCC1) was necessary, to avoid possible cold zones that would compromise complete microbiological inactivation, in correlation with other results (Sumithra & Bhattacharya, 2008).

Continuing the technological process, after the heat treatment stage, critical control point 2 (PCC2) is established, which targets the deep freezing phase of the semi-finished product. This control aims to guarantee microbiological stability and extend the shelf life, by reaching an internal temperature of at least −18.5°C in the center of the product. Respecting this critical limit is essential to prevent the development of post-processing residual microflora and to maintain the structural integrity of the product during storage and distribution, in accordance with the results of other studies (Kataoka et al., 2017). Comparatively, the three analyzed variants differ in their freezing behavior, directly influenced by the composition and texture of the coating. Products coated with corn flakes, having an expanded and aerated structure, facilitate rapid thermal diffusion, which allows reaching the critical temperature in a shorter time. In contrast, products coated with pumpkin seeds have a denser and more compact crust, associated with lower thermal conductivity, which determines a greater thermal inertia and requires an extension of the freezing time, according to the product's technical specification.

This adjustment is justified by the crust's structural density, which slows down heat transfer and requires prolonged exposure to the freezing environment to meet microbiological safety standards. The variant coated with classic breadcrumbs is located between the two, with a stable and predictable thermal behavior. These observations justify adjusting the technological parameters depending on the type of coating, to ensure compliance with the requirements established at this control point.

After freezing, the product is subjected to verification through critical control point 3 (CCP3), corresponding to metal detection. This step has the role of eliminating the risk of physical contamination with metal fragments from processing or handling equipment, such as knives, conveyor belts or fasteners. Detectors calibrated for ferrous, non-ferrous and stainless steel are programmed to automatically identify and reject any product that exceeds the preset sensitivity threshold (minimum 3.5 mm) (Liu & Zhou, 2011). Although the detection system works uniformly regardless of the product, the type of coating used can indirectly influence the efficiency of this step. Products coated with pumpkin seeds or corn flakes generate a more voluminous crust, with variable density, which can affect, in rare cases, the accuracy of detection by attenuating the electromagnetic signal. In addition, these ingredients pose an increased risk of cross-contamination, especially for people with food allergies, which is why cleaning and validation of equipment become more demanding after processing these variants. Therefore, the frequency of testing with standards and rigorous documentation of maintenance interventions become imperative to maintain compliance at this critical point. Each stage controls specific risks, from the inactivation of pathogens, to microbiological stabilization over time and the prevention of physical contamination. Structural differences

generated by the type of coating applied (breadcrumbs, corn flakes, pumpkin seeds) influence the behavior of the products in relation to these critical parameters, requiring fine technological adaptations to maintain compliance and quality standards.

Table 1 presents the critical control points identified in the production process, highlighting the main objectives, the associated critical limits and how the type of coating influences each stage, with significant differentiations between the products analyzed.

Table 1. Critical control points and the influence of the type of coating

Critical Control Point	Main Objective	Critical Limit	Differentiated Impact Between Products
CCP1 – Thermal treatment	Inactivation of pathogenic agents (*Salmonella* spp., *Listeria monocytogenes*)	≥ 72°C at the core of the product, maintained for at least 26 seconds	The dense crust of the pumpkin seed variant requires additional monitoring of internal temperature
CCP2 – Freezing	Microbiological stabilization by reaching freezing temperature	≤ −18.5°C at the core of the product	Cornflakes allow for faster freezing; pumpkin seeds require extended time for complete cooling
CCP3 – Metal detection	Prevention of physical contamination with metal fragments from equipment	Detection of particles ≥ 3.5 mm (Fe, non-Fe, stainless steel)	Irregular crust (corn, pumpkin) may affect detection sensitivity; requires more frequent testing

In all three products, the raw material used is the inner fillet of chicken breast, a fine muscle with a high protein content (22-24 g/100 g) and a low lipid intake. Due to its tender structure and lack of intramuscular fat, this type of meat favors the absorption of marinades, but is susceptible to dryness during heat treatment. The final texture is significantly influenced by the type of coating applied and the processing parameters (Almeida et al., 2015).

Table 2 presents the analysis of the nutritional values of the three semi-finished products made from chicken fillets, coated with classic breadcrumbs, corn flakes and pumpkin seeds, respectively, highlighting significant differences in chemical composition and energy value per 100 grams of product. These variations are mainly attributable to the differences between the types of coatings used, but also to the degree of processing and the auxiliary ingredients included in the recipe.

Table 2. Nutritional comparison between the three variants of semi-prepared products (per 100 g)

Parameter	Classic Breadcrumbs	Cornflakes	Pumpkin Seeds
Energy value (kcal)	250	215	228
Fats (g)	9.4	8.5	11
Saturated fats (g)	–	0.9	2.2
Carbohydrates (g)	27.6	19	12
Sugars (g)	0.7	0.7	1.0
Proteins (g)	13.9	15.5	19
Fibers (g)	–	–	1.5
Salt (g)	1.0	1.3	0.8

The product coated with classic breadcrumbs is notable for its relatively high energy value (250 kcal/100 g), reflecting a higher carbohydrate intake (27.6 g) and a moderate lipid content (9.4 g). Proteins reach a level of 13.9 g, corresponding to a balanced protein density for a standardized bread product. This variant maintains a familiar composition, with added vegetable fibers and basic ingredients (wheat flour, yeast, rapeseed oil), without a significant intake of ultra-processed additives. The product coated with corn flakes presents a lighter nutritional profile in terms of calories (215 kcal/100 g), with a clear reduction in the total amount of carbohydrates (19 g), but also with a higher protein content (15,5 g), which makes it more suitable for a balanced hypocaloric diet. The presence of dextrose, glucose syrup and vegetable protein hydrolysate can contribute to improving the technological and sensory profile, but implies a higher degree of processing. The salt content (1.3 g) is the highest of the three products, being influenced by the multiple additions in the composite breadcrumbs. In contrast, the product coated with pumpkin seeds is distinguished by a higher protein intake (19 g/100 g) and a minimum carbohydrate content (12 g), while being the only product

that naturally contains dietary fiber in a notable percentage (1.5 g). With a moderate caloric intake (228 kcal), this product combines the nutritional advantages of chicken fillet with those of oilseeds, rich in unsaturated fatty acids and bioactive compounds. At the same time, it is the product with the lowest salt content (0.8 g), which makes it recommended for low-sodium diets. The comparative analysis of the nutritional values of the three semi-prepared products made of chicken fillets covered with classic breadcrumbs, corn flakes and pumpkin seeds highlights important differences, influenced both by the type of outer layer applied and by the degree of processing of the ingredients used, correlated with specialized studies (Bourn & Prescott, 2010; Marin, 2017; El-Kewaiey, 2012). The product covered with classic breadcrumbs presents a traditional composition, with a relatively high caloric intake and a high carbohydrate content. In contrast, the cornflakes variant stands out for a lower caloric profile and a moderate to high protein content, but the advanced degree of processing and the increased salt content may constitute limiting factors in the context of a balanced diet. On the other hand, the product coated with pumpkin seeds is distinguished by the most favorable nutritional composition, reflected in the higher protein intake, low carbohydrate and salt content, as well as the presence of dietary fiber and beneficial unsaturated fats. Thus, this variant can be considered the most advantageous from a nutritional point of view, addressing especially consumers interested in functional products and diets oriented towards metabolic health.

In order to confirm the compliance and food safety of the semi-finished product made from chicken fillet, coated with corn flakes, a series of laboratory analyses were carried out, covering the three main risk categories: microbiological, chemical and specific contaminants (mycotoxins, heavy metals, residues).

The results indicated:

• *Salmonella* spp.: not detected in 25 g of product, in all subsamples tested, according to ISO 6579-1:2017. According to the legislation, this bacterium should not be present in partially heat-treated meat products;

• *Escherichia coli, NTG, yeasts and molds*: below the detection limit ($<1x10^1$ CFU/g), indicating appropriate hygiene in the manufacturing process;

• *Coagulase-positive staphylococci*: absent (<10 CFU/g), according to ISO 6888-1, which reflects the lack of contamination with pathogenic flora specific to improper meat handling.

These results are within the limits allowed by European legislation, which provides, for example, a maximum limit of 10^2 CFU/g for *E. coli* in products of this type and zero tolerance for *Salmonella* spp. (Ehuwa, 2021). Thus, from a microbiological point of view, the analyzed product is considered appropriate and safe for consumption.

In addition to microbiological safety, analyzes were also carried out for chemical contaminants, in particular mycotoxins and residual disinfectant compounds (chlorate and perchlorate), specifically applicable to the vegetable components of the crust, in this case, corn flakes.

The results were as follows:

• Aflatoxins B1, B2, G1, G2 and total: all below the detection limit (<0.1 µg/kg), while the maximum legal limit for food is 2.0 µg/kg for B1 and 4.0 µg/kg for total, according to Regulation (EU) 915/2023;

• Fumonisins B1 and B2: <30 µg/kg, with the regulatory limit of 400 µg/kg in cereals and derived products, the result being considerably below this value;

• Zearalenone: <20 µg/kg, compared to the maximum permitted limit of 75-100 µg/kg, depending on the product;

• Chlorate and perchlorate: <0.010 mg/kg, below the limit of toxicological interest (TDI – tolerable daily intake) set at 0.01 mg/kg body weight for chlorate and 0.002 mg/kg body weight for perchlorate, which suggests the absence of contamination with residues from sanitizing substances or contaminated water.

All these results demonstrate that the product is within safety limits, indicating the use of clean raw materials, controlled processing and the absence of contamination from industrial or agricultural sources (El-Kewaiey, 2012; Andrade et al., 2018; Castells et al., 2008; Chaunier et al., 2005).

For the semi-finished product of chicken fillet, covered with a layer of pumpkin seeds, laboratory analyses were carried out to verify microbiological and chemical compliance, as well as the absence of contaminants specific to oleaginous raw materials. The related analysis bulletins cover both the final product (prepared and frozen) and the vegetable component used as breading (pumpkin seeds as such).

The testing was carried out on five distinct sub-samples, and the results confirmed the complete absence of this bacterium in all cases analyzed. Considering that the product is a semi-prepared product, partially heat-treated, but intended for an additional baking or frying step by the consumer, the presence of *Salmonella* spp. would be unacceptable (Ehuwa, 2021). The legislation requires the absence of this bacterium in 25 g of product for all incompletely cooked foods of animal origin. Therefore, the result obtained is in accordance with current food safety requirements, reflecting a hygienic and well-controlled production process.

Considering the oleaginous nature of pumpkin seeds (Leichtweis et al., 2025), laboratory analyses were performed to detect Ochratoxin. A mycotoxin frequently associated with such ingredients. The determination was performed using liquid chromatography with triple quadrupole detection (LC-QQQ), considered an international standard for sensitivity and accuracy. The result indicated a concentration below the detection limit of the method (<1 µg/kg). This is a very low value, in the context of which the maximum limit allowed by European legislation for oilseeds is 5 µg/kg (according to Regulation (EU) No. 915/2023). Thus, the result obtained not only complies with legal limits, but also indicates a superior quality of the raw material, demonstrating that the pumpkin seeds used were not exposed to improper storage conditions or fungal contamination, results also highlighted by other studies (Fatima et al., 2025).

Based on the results of the available analyses, the semi-finished product with pumpkin seed crust can be considered safe for consumption from a microbiological and chemical point of view. The absence of *Salmonella* spp. in the final product, corroborated with the very low levels of mycotoxins in the vegetable component, confirms that the manufacturing process was carried out under conditions compliant with legislative requirements and good practices in the food industry.

In order to validate the quality and health safety of the classic variant of the semi-finished product based on chicken fillets with a white breadcrumb coating, a series of physico-chemical and microbiological analyses were carried out, applied both to the final product and to the component raw materials - meat, breadcrumbs, flour. These analyses comply with the regulations established by Regulation (EC) No. 2073/2005 on microbiological criteria, as well as with Regulation (EU) No. 915/2023 on chemical contaminants in food.

The results showed:

• *Salmonella* spp.: not detected in all 5 subsamples, according to ISO 6579-1:2017;

• *E. coli*, coliforms, yeasts, molds: below 10 CFU/g - well below the guideline limits allowed;

• *NTG*: 1.3 x 10^2 CFU/g - value considered very good for bakery products, according to GHP (Good Hygiene Practice) standards.

The breadcrumbs were tested for acrylamide content, a potentially toxic substance formed by Maillard reactions during baking at high temperatures. The acrylamide level was <10 µg/kg, significantly below the reference level of 50 µg/kg established by Regulation (EU) 2017/2158 for bakery products, indicating a well-controlled baking process and the use of appropriate raw materials (Mesias et al., 2023).

The analysis of the mycotoxin DON, in correlation with specialist studies (Ji et al., 2024), one of the most common in cereals, was carried out on the white breadcrumbs. The result was <14 µg/kg, compared to the maximum permitted limit of 500 µg/kg for bread and cereal-derived products. This result denotes insignificant contamination, within excellent safety limits. Regarding the flour used in the first crumbing stage, the analysis included determinations for aflatoxins B1, B2, G1, G2, zearalenone and ochratoxin A. All values were below the limits of quantification (B1-B2-G1-G2 <0.5 µg/kg, zearalenone <20 µg/kg, ochratoxin A <1 µg/kg), reflecting a safe raw material, originating from controlled supply chains. The determination of the moisture content of the white breadcrumbs

indicated an average value of 6.25 g/100 g, with an uncertainty of ±0.28. Low moisture contributes significantly to the microbiological stability and the crispy texture of the final crust (Silva de Lana et al., 2025).

The hygienic and sanitary quality of the raw material was confirmed by physico-chemical analyses, in particular by determining the content of heavy metals. The values obtained for lead (<0.03 mg/kg) and cadmium (<0.01 mg/kg) were well below the maximum limits allowed by European legislation for poultry meat (0.10 mg/kg for lead and 0.05 mg/kg for cadmium), which attests to the absence of contamination with toxic metals and the suitability of the raw material for food safety (Rebezov et al., 2021).

In addition to these determinations, other essential indicators for evaluating the quality of refrigerated meat are pH and readily hydrolyzable nitrogen, the variation of which is directly correlated with storage time. Increased values of these parameters are associated with biochemical degradation of proteins and, implicitly, with decreased freshness, representing reliable benchmarks for establishing the consumability threshold (Surmei & Usturoi., 2012).

In order to evaluate the *sensory characteristics* of the three product variants, they were subjected to a standardized final heat treatment, consisting of baking in an electric oven at a temperature of 180°C, for 30 minutes. This stage aimed to simulate the home preparation method, under reproducible conditions, allowing an objective assessment of the organoleptic differences between the products.

For carrying out sensory evaluation, participants were previously instructed on how to complete the questionnaire and the evaluation criteria, and the products were graded A, B, C, for easier scoring. Each participant rated the three products based on four fundamental sensory attributes: visual appearance, texture, taste and aroma, using a hedonic scale from 1 to 9, where 1 indicated the minimum level of pleasure and 9 the maximum. The results obtained highlighted differences between the variants.

Figure 1 illustrates the average scores obtained for four sensory attributes: appearance, texture, taste and aroma, in the comparative evaluation of three variants of semi-finished products, differentiated by the type of coating: classic breadcrumbs, corn flakes and pumpkin seeds.

In the sensory analysis, variant A, made with classic breadcrumb coating, recorded moderate and constant scores for all evaluated attributes, ranging from 6.81 to 7.10. These results indicate a high level of acceptability, but without remarkable characteristics that would clearly differentiate it from the other variants.

The use of standard breadcrumbs is integrated into the preferences of the average consumer, being associated with familiar and widely distributed products on the market.

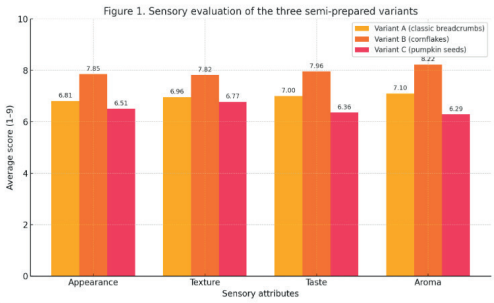


Figure 1. The impact of crust type on the sensory perception of semi-finished products

This familiarity generates a perception of stability and taste predictability, which can support consumer loyalty, but can also limit perceived innovation. The texture of the product was considered adequate, but without offering a distinct experience, which may explain its more modest positioning in the preferences expressed by the participants.

Variant B, covered with corn flakes, was clearly the best appreciated from a sensory point of view, obtaining the highest average scores on all four criteria analyzed, 7.82-8.22. It was particularly noted for its attractive appearance, golden crust and crispy texture, elements that, together, determined a pleasant and memorable consumption experience. The taste was described as balanced, with discreet notes of sweetness and toastiness, and the intense aroma completed the sensory profile in a harmonious way. The success of this variant can be attributed both to its technological qualities and to the fact that corn flakes are a known and accepted ingredient by the general public, often associated with crispy and

appetizing products. The flakes also contribute to the formation of an aerated crust, with a low degree of compaction, which enhances the perception of freshness and lightness when chewing.

In contrast, variant C, with a pumpkin seed coating, obtained lower scores than the other two variants, especially in the taste and aroma attributes, 6.29-6.51. Although the texture was appreciated by some participants as interesting and different, the aromatic profile proved more difficult to accept. This result can be correlated with the low level of consumer exposure to such combinations.

The acceptability of unconventional ingredients is often influenced by eating habits and individual taste repertoire. In this case, pumpkin seeds, although beneficial from a nutritional point of view, are less often used as a coating for semi-prepared products, which may generate an initial reluctance in acceptance. Therefore, the scores obtained do not necessarily reflect a poor sensory quality, but rather a gap between innovation and the level of familiarity of the public with the respective ingredient.

The distribution of preferences expressed by the participants was consistent with the quantitative data: 50% of the tasters indicated the cornflakes variant as their favorite, 33.33% opted for the pumpkin seed variant, and 16.67% chose the product covered with classic breadcrumbs. These results suggest that, although traditional products remain appreciated for their simplicity and safety, moderate innovations, such as cornflakes, can bring added attractiveness, while more avant-garde solutions, such as the use of seeds, require additional communication and consumer education strategies to be fully exploited.

CONCLUSIONS

The comparative study conducted on the three variants of semi-finished products based on chicken fillets, with classic breadcrumb coating, with corn flakes and with pumpkin seeds, highlighted significant differences from a technological, sensory and nutritional point of view.

From the perspective of technological parameters, it was found that the type of coating directly influences the behavior of the product during heat treatment, oil absorption, moisture retention and the efficiency of critical control points (CCP). The variant with corn flakes presented the most balanced behavior during processing, associating a crispy crust with good thermal penetration and efficient freezing, while the pumpkin seeds determined a denser crust and increased thermal inertia, requiring time and temperature adjustments to guarantee microbiological safety. The nutritional analysis revealed a superior profile in the case of the pumpkin seed product, characterized by the highest protein content (19 g/100 g), a reduced intake of carbohydrates and salt, as well as the presence of dietary fiber and unsaturated fats, parameters that recommend it for a functional and balanced diet. In contrast, the classic breadcrumb version stood out for its conventional, familiar composition, but with a higher caloric and carbohydrate intake, without innovative functional elements.

From a food safety point of view, all three products fell within the limits set by European legislation, both in terms of microbiological criteria and chemical contaminants (mycotoxins, heavy metals, residues). The absence of pathogens such as *Salmonella* spp. and very low levels of potentially toxic substances confirm the conformity of the technological process and the quality of the raw materials used. The sensory evaluation demonstrated a clear consumer preference for the cornflakes product, which obtained the highest scores in terms of visual appearance, crispy texture and taste balance. The classic breadcrumb variant was appreciated for its familiarity, but did not highlight any distinctive characteristics, while the pumpkin seed product, although nutritionally valuable, encountered reluctance from consumers unfamiliar with this texture and flavor.

Therefore, the integration of innovative plant ingredients into the structure of semi-finished poultry products proves feasible, both from a technological and food safety point of view, provided that the process is correctly adapted and the market is positioned appropriately.

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HARNESSING BLOCKCHAIN FOR ENHANCED RISK MANAGEMENT IN THE FOOD SUPPLY CHAIN

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Abstract

The paper aimed to present the potential of blockchain technology in enhancing food safety and transparency across the global food supply chain. Blockchain's decentralized, immutable ledger enables end-to-end traceability of products, addressing critical issues of food fraud, contamination, and inefficiency. Through case studies, including IBM Food Trust initiatives at Walmart and Carrefour, this research demonstrates how blockchain can streamline traceability, improve operational efficiency, and bolster consumer trust by providing access to verified information on product origins. Despite these benefits, challenges such as high implementation costs, interoperability issues, and regulatory concerns remain barriers to widespread adoption. This paper offers insights and recommendations to facilitate blockchain's integration in the food industry, advancing toward a safer, more transparent, and sustainable food system.

Key words*: control, digital ledger, food safety, quality, traceability.*

INTRODUCTION

As the world faces rapid population growth, maintaining food safety has become a global priority.

The global population is projected to surge to 8.5 billion by 2030 and 9.7 billion by 2050 (United Nations, 2022).

This growth places immense pressure on the global food supply chain, which must maintain food quality, ensure safety, and minimize waste.

The food supply chain, a complex network of stakeholders, faces challenges like fraud, contamination, and inefficiencies, demanding innovative solutions for traceability and transparency. Blockchain technology, with its decentralized and immutable ledger system, offers potential for solving these issues. By enabling detailed and tamper-proof records from farm to table, blockchain enhances safety, trust, and operational efficiency, while addressing concerns of scalability, interoperability, and privacy (Crosby et al., 2016).

The purpose of this research is to explore how blockchain technology can be leveraged to mitigate risks in the food supply chain, with a particular focus on enhancing traceability to prevent biological, chemical, and mechanical contamination. This study aims to investigate the implementation and benefits of blockchain-based traceability systems in ensuring food safety, improving supply chain transparency, and fostering consumer trust.

MATERIALS AND METHODS

This study uses a mixed-methods approach, combining case studies with thematic analysis to assess blockchain's impact on food safety and traceability.

A digital traceability system enables farmers to monitor and record every stage of their produce lifecycle, from planting to delivery, ensuring transparency and data integrity.

Case studies, such as Walmart or Pietro Coricelli, take us behind the scenes of these digital transformations, uncovering the complexity and advantages of blockchain implementation.

Data sources include case studies from IBM Food Trust, Walmart, and Carrefour, along with industry reports, academic publications, and government regulations (International Business Machines Corporation, n.d.).

Primary data is gathered through interviews with stakeholders involved in blockchain implementations, while secondary data is drawn from existing reports and regulatory documents.

Thematic analysis will identify key trends, challenges, and best practices, providing a comprehensive understanding of blockchain's role in mitigating risks in the food supply chain.

RESULTS AND DISCUSSIONS

Blockchain technology has emerged as a transformative force in the food supply chain, enhancing traceability, transparency, and operational efficiency.

Blockchain revolutionizes data management by introducing distributed ledger systems (Casino et al., 2019).

While centralized systems (Figure 1, left) rely on a single point of failure, blockchain (Figure 1, right) creates a peer-to-peer network where data is replicated across multiple nodes. This decentralized architecture makes blockchain more resilient to attacks and more transparent.

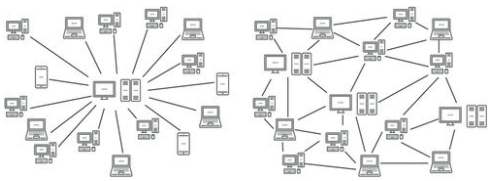


Figure 1. Difference between Centralized (left) and Distributed database (right) (Patelli & Mandrioli, 2020)

Traceability

In real-world scenarios, such as mango tracking at Walmart, blockchain turns a seven-day search into a process lasting only a few seconds (International Business Machines Corporation, n.d.).

This efficiency saves not just time but also resources, ensuring that potentially contaminated products are swiftly removed from circulation.

Such speed is critical in mitigating the impact of foodborne illnesses.

Transparency

For companies like Pietro Coricelli, blockchain authenticates the journey of olive oil from grove to shelf. Consumers can access verifiable details about the product's origin, production processes, and quality standards.

This level of transparency combats counterfeiting and builds trust.

Operational Efficiency

By using blockchain to monitor storage and transport conditions in real time, companies such as Antonello Produce significantly reduce waste while maintaining product freshness. This optimized logistics framework ultimately enhances customer satisfaction and cuts costs.

Stakeholder Impact

The benefits of blockchain adoption extend across the food supply chain, impacting various stakeholders (Hughes et al., 2019):

- ***Producers***: blockchain improves brand reputation and market differentiation by offering transparent product information. For example, Pietro Coricelli and Antonello Produce have strengthened consumer trust and brand value through blockchain-enabled traceability.
- ***Distributors***: enhanced tracking capabilities minimize product losses and streamline compliance with regulatory standards. Farmer Connect demonstrates how blockchain facilitates efficiency by ensuring direct connections between coffee farmers and consumers.
- ***Retailers***: reliable and transparent data builds consumer confidence. Walmart's use of blockchain to verify product origins has enhanced customer trust and reduced risks associated with counterfeit or unsafe goods.
- ***Consumers***: access to trustworthy, detailed product information empowers informed decision-making, improving consumer satisfaction and loyalty.

Companies like Walmart, Pietro Coricelli, Antonello Produce, and Farmer Connect illustrate the successful adoption of blockchain technology through IBM's Food Trust platform (Table 1).

Addressing Challenges

Despite its promise, blockchain is not without challenges (Ismail & Materwala, 2019).

The challenges in blockchain adoption for supply chains requires tackling high costs, scalability issues, data privacy concerns, and regulatory barriers:

- ***High costs***: The initial investment and ongoing maintenance expenses can deter smaller organizations from adopting blockchain solutions;

- ***Scalability issues***: Integrating blockchain with complex, high-volume supply chains present scalability challenges. Innovations in blockchain architecture, such as Layer 2 solutions, may mitigate these issues;
- ***Data privacy***: Sharing sensitive supply chain information on a blockchain network raises concerns about data privacy and confidentiality. Employing advanced encryption techniques and permissioned blockchains can address these concerns;
- ***Regulatory barriers***: The evolving nature of food safety regulations poses challenges for blockchain adoption. Industry-wide collaboration is crucial to establish standard protocols and ensure compliance with international guidelines.

By leveraging innovative solutions such as Layer 2 architectures, advanced encryption, permissioned blockchains, and fostering industry-wide collaboration, organizations can overcome these obstacles and unlock the transformative potential of blockchain technology (Cachin & Vukolic, 2017).

Table 1. Key Impacts of Blockchain Adoption

Company	Industry	Blockchain Application	Key Impacts	Source
Pietro Coricelli	Olive Oil	Traceability from grove to shelf	Increased consumer trust, brand differentiation	https://www.ibm.com/us-en
Antonello Produce	Fresh Produce	Tracking the journey of produce	Improved safety, reduced waste, enhanced reputation	https://www.ibm.com/us-en
Farmer Connect	Coffee	Connecting farmers and consumers	Promoted fair trade, increased transparency	https://www.ibm.com/us-en
Walmart	Retail	Tracking perishable goods like mangoes	Enhanced recall efficiency, consumer confidence	https://www.ibm.com/us-en

Practical Applications and Future Prospects

The ability of blockchain to integrate with complementary technologies such as the Internet of Things (IoT) and cloud computing makes it an invaluable tool for supply chain optimization through (Kim & Laskowski, 2018; Wang et al. 2019):

- ***Real-Time monitoring***: sensors linked to blockchain systems provide continuous updates on product conditions, enabling proactive decision-making;
- ***Predictive analytics***: blockchain-powered data analytics tools predict supply chain disruptions, enhancing resilience and efficiency.

Blockchain has the potential to greatly improve transparency and efficiency in global food supply chains. By enabling real-time tracking and providing immutable records, it offers a powerful tool to address fraud and contamination issues. Studies suggest that blockchain can significantly enhance transparency, ensuring that consumers and stakeholders have access to verified, traceable product information (Pandey et al., 2022).

Food traceability can be analyzed through both user interfaces and the underlying blockchain infrastructure. The process begins with a user-friendly dashboard that provides access to key traceability data. From there, the system relies on a decentralized network of nodes and an immutable digital ledger to securely record every transaction within the supply chain, ensuring transparency and food safety.

For example, in coffee supply chains, blockchain technology enables end-to-end traceability, allowing stakeholders to track the journey of coffee beans from their origin to the final consumer (Figure 2).

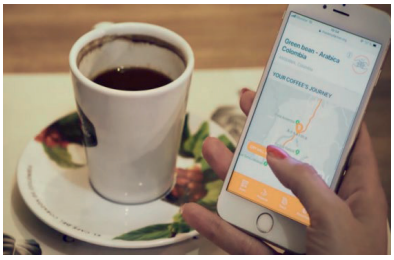


Figure 2. Traceability of coffee beans using Farmer Connect with IBM Food Trust Blockchain Network (https://www.ibm.com/us-en)

A Whole Grain Margherita Pizza consists of ingredients sourced from multiple locations across the global supply chain. Ensuring the authenticity and quality of these ingredients

requires a robust traceability system. Blockchain technology facilitates this process by providing transparent and verifiable data on the origin, production, and distribution of each component (Figure 3).

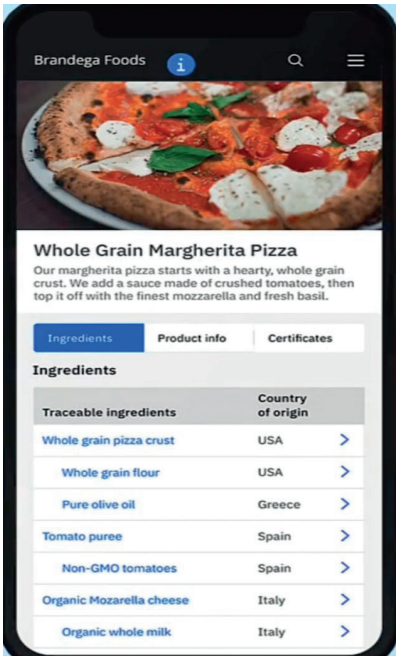


Figure 3. Traceability for Margherita Pizza from Brandega (https://www.ibm.com/us-en)

Exploring the Blockchain Architecture: Insights from MultiversX

Blockchain technology serves as the backbone of modern traceability solutions, transforming how we ensure transparency and trust in the food supply chain. Understanding the role of blockchain in traceability requires an examination of the MultiversX infrastructure - a blockchain that can be utilized to record every detail of a product's journey from origin to destination. Using data from the MultiversX testnet, this section explores how transactions are recorded, verified, and linked to create an immutable chain of information. Additionally, it highlights the critical function of nodes within the distributed network, ensuring data integrity and security.

The Blockchain's Role

Blockchain acts as an immutable digital ledger, documenting every transaction and movement of ingredients from farm to table. Each step in a product's journey is stored in a block, and these blocks are linked together, forming a secure and unalterable chain of information.

By enabling decentralized operations and secure data-sharing, blockchain allows companies to operate more collaboratively, reducing inefficiencies and fostering innovation. This shift in business models is particularly evident in industries like food supply chains, where blockchain's transparency and security are pivotal for building trust and improving operational practices (Scott et al., 2017).

Nodes: The Backbone of the Network

Unlike traditional databases, blockchain is not stored in a single location, it operates across a decentralized network of computers called nodes. Each node maintains a complete copy of the blockchain, contributing to its resilience and security.

Our TestNet wallet on the xSpotlight platform is a gateway to the exciting world of blockchain development. Built on the MultiversX blockchain, this platform empowers us to experiment and learn. This section examines the potential of decentralized applications and their role in supporting the growth of blockchain technology (Figure 4).

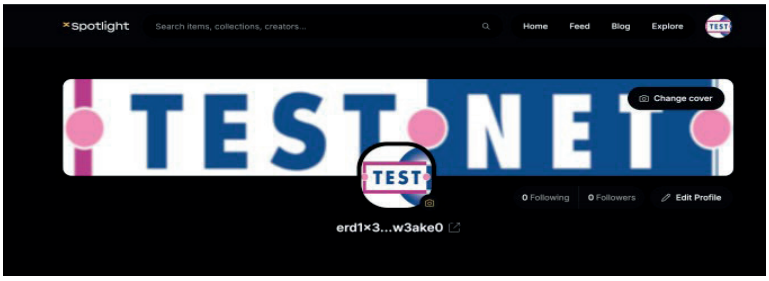


Figure 4. TestNet Traceability Blockchain Network thru xSpotlight using MultiversX Blockchain, made in Romania (Original)

Displayed is a transaction record from the MultiversX Testnet Explorer. It offers a glimpse into the underlying technology of blockchain, where every transaction is recorded and verified on a distributed ledger.

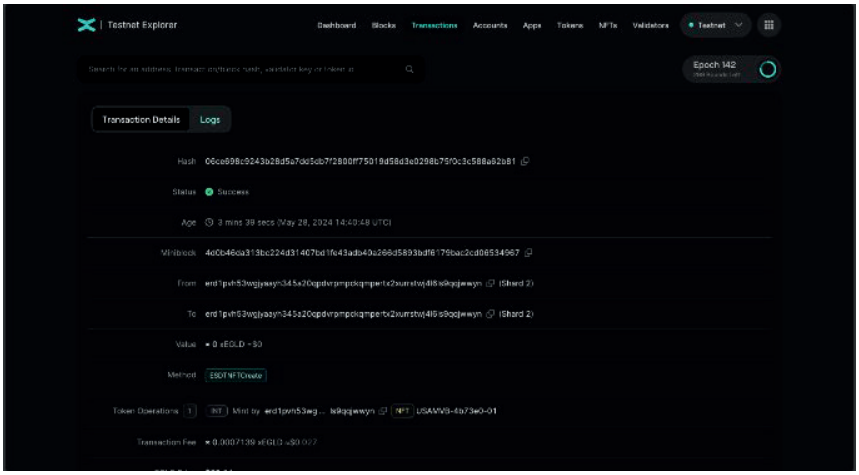


Figure 5. Transactions details on MultiversX Testnet Explorer (Original)

The provided data, such as the transaction hash, timestamp, and involved parties, demonstrates the transparency and security inherent in blockchain networks (Figure 5).

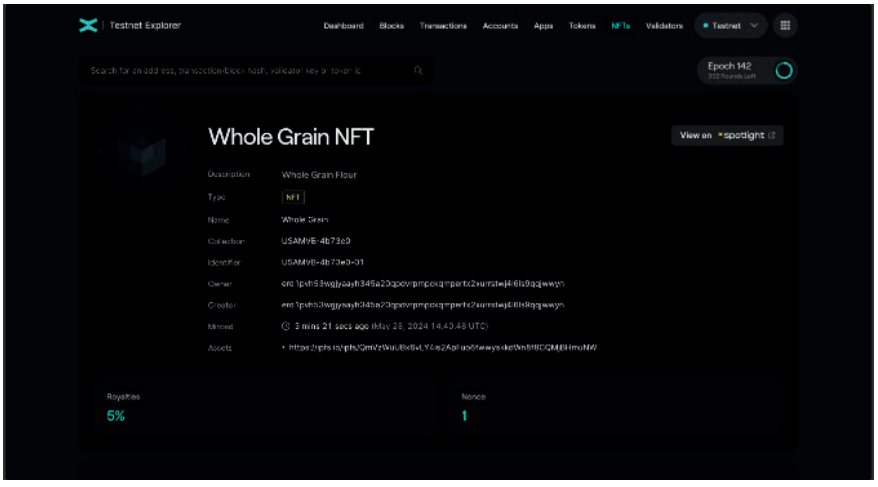


Figure 6. Information on its creator, the timestamp on Testnet Explorer (Original)

Figure 6 captures a snapshot of a Whole Grain on the MultiversX Testnet. The image provides detailed information about the product, including its creation timestamp, creator's wallet address, and associated metadata.

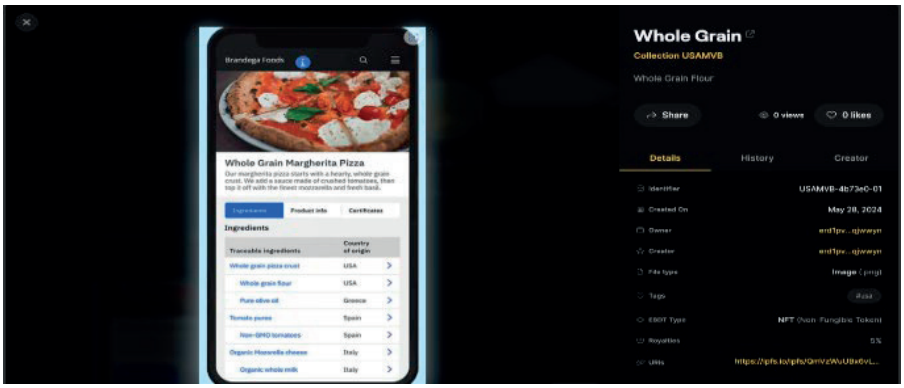


Figure 7. Margherita Pizza from Brandega Food in Blockchain on xSpotlight platform (Original)

Figure 7 depicts a digital representation of a Margherita Pizza on the xSpotlight platform. The Image associated with this pizza provides a detailed breakdown of its ingredients, their origin, and the entire production process. By utilizing blockchain technology, Brandega Food ensures the integrity and transparency of their products, allowing consumers to make informed choices about the food they consume.

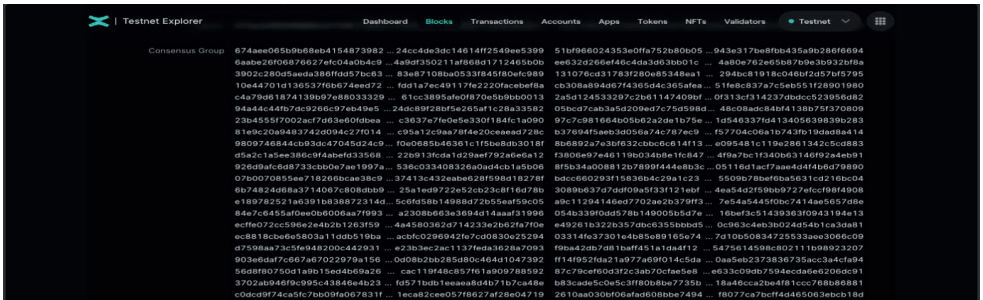


Figure 8. All validators “People” who add info to the block “Margherita Pizza” on Testnet Explorer MultiversX Blockchain (Original)

Figure 8 illustrates the collaborative effort of validators in securing the MultiversX Testnet. These validators, represented by their unique addresses, work together to verify and add new blocks to the chain, including the one containning the Margherita Pizza. This collective effort ensures the integrity and security of the network, making it a reliable platform for various applications, including food traceability. Behind the scenes, blockchain technology underpins the app's functionality. It records each step of the Margherita pizza's journey, from sourcing the fresh ingredients - such as tomatoes, mozzarella, and basil - to dough preparation, baking, and packaging. This data is stored in immutable blocks, creating a transparent and tamper-proof record. Each participant in the supply chain, such as farmers, processors, and distributors, acts as a node in the blockchain network. They contribute data about the movement and processing, which is verified and added to the blockchain. This ensures the information is accurate and trustworthy (Gipp et al., 2015).

Blockchain technology, along with its network of nodes and complex data structures, may initially appear sophisticated. The numerous technical terms and interconnected processes can create a perception of complexity. However, these elements represent the

underlying framework that ensures transparency, security, and traceability within the system.

From Complexity to Simplicity: Navigating Blockchain Through IBM Food Trust

The IBM Food Trust Platform serves as a user-friendly interface designed to enhance traceability within the supply chain. It simplifies the complexity of blockchain technology by offering an intuitive dashboard, where raw data is transformed into actionable insights. Through features such as visual analytics, real-time tracking, and automated data integration, the platform facilitates informed decision-making for businesses aiming to improve transparency and operational efficiency.

While blockchain provides the underlying infrastructure for secure data management, IBM Food Trust offers a structured environment where stakeholders can monitor and verify product traceability with ease. The following section explores the dashboard's functionalities and its role in streamlining supply chain oversight (Figure 9).

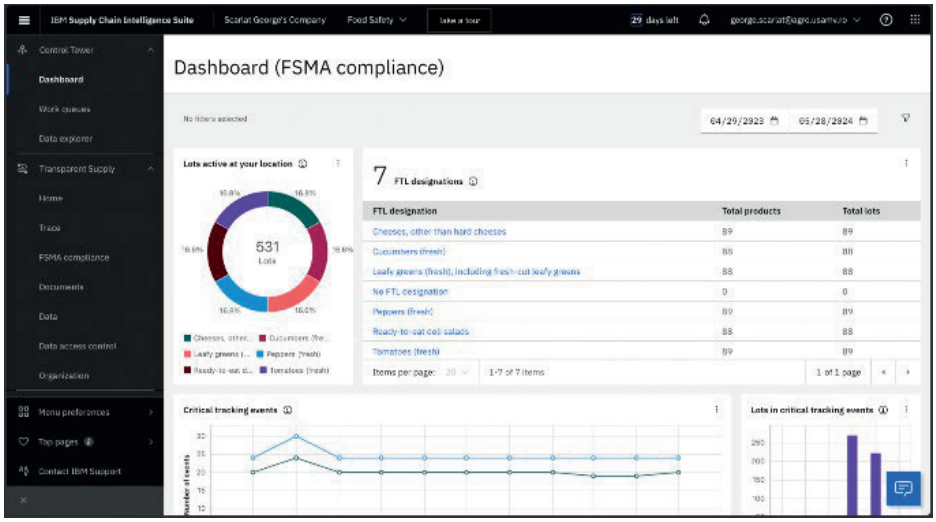


Figure 9. IBM Supply Chain Intelligence Suite Dashboard (Original)

Core components of the dashboard are represented by:

- Dashboard (FSMA compliance): The main title indicating the dashboard's focus.
- Work queues: A section likely for managing tasks and alerts related to food safety.
- Data explorer: A tool for exploring and analysing data within the system.
- Transparent Supply: A module that provides visibility into the supply chain.
- Lots active at your location: A counter showing 7 active lots.
- FTL designations: A table listing different food types and their corresponding FTL (Full Truckload) designations, along with total products and lots.
- Lots: A pie chart illustrating the distribution of lots across various food categories.
- Critical tracking events: A graph showing the trend of critical events over time.

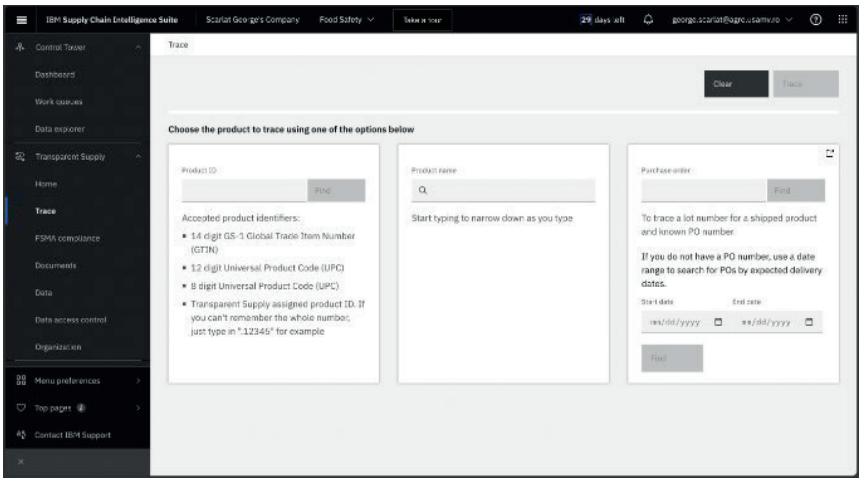


Figure 10. IBM Food Trust Platform: Simplifying Traceability with the Data Explorer (Original)

IBM Food Trust Platform's Data Explorer section, a prime example of how technology simplifies complexity. Here's how it translates the intricate blockchain data into an organized, user-friendly interface (Figure 10), like:

- ***Organized Data***: The platform neatly arranges information into columns: Lot Code (a unique identifier), Type (product category), Product Name, Product ID, Lot Code Creator, Lot Origin Location, and more. This clear structure makes it easy to locate specific products and track their journey through the supply chain.
- ***Searchable and Filterable***: The search bar at the top allows users to quickly find specific lot codes or products. Additionally, the platform likely offers filtering options to narrow down the results by type, location, or other relevant criteria.
- ***Transparency at a Glance***: Each row represents a single lot of a product. By clicking on a lot code, users can access detailed information about its origin, processing, and movement throughout the supply chain. This transparency empowers stakeholders to verify the authenticity and quality of products.
- ***Streamlined Collaboration***: The platform is designed for collaboration among different stakeholders in the supply chain. Suppliers, manufacturers, distributors, and retailers can share data securely, ensuring everyone has access to the most up-to-date information.

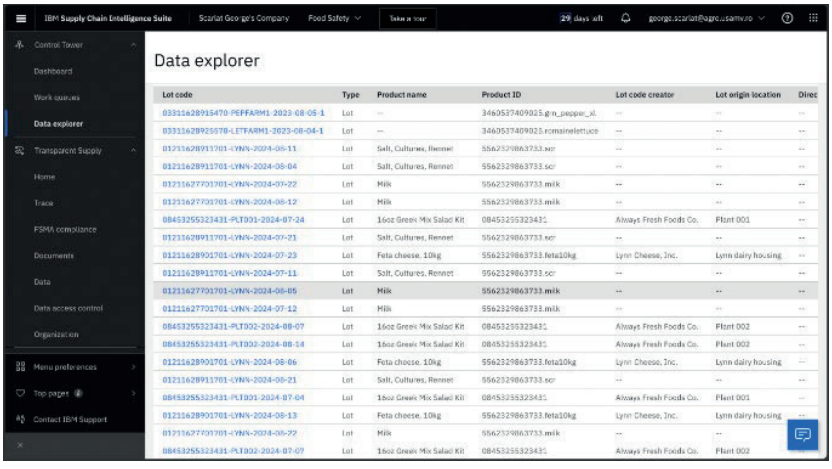


Figure 11. Trace feature on IBM Dashboard (Original)

This image displays the "Trace" feature within the IBM Supply Chain Intelligence Suite. This interface allows users to easily trace the journey of a product throughout the supply chain (Figure 11).

Here's how it simplifies the complexity of traceability:

Multiple Search Options: The platform provides three distinct search options, accommodating different ways users might identify a product:

- ***Product ID***: This could be an internal identifier assigned by the company or a universal code like a Global Trade Item Number (GTIN) or Universal Product Code (UPC);
- ***Product Name***: Users can search by the product's name, which is useful when the exact ID isn't known;
- ***Purchase Order (PO) Number and Date Range***: If the user knows the purchase order associated with the product, they can input that along with a date range to narrow down their search.

User-Friendly Search: The platform allows users to start typing and automatically narrows down the search results, similar to a search engine, making it easier to find the right product even with partial information.

Clear Instructions: Clear instructions are provided for each search option, explaining what information to enter and how to use the search effectively.

This helps even users unfamiliar with the platform to use it efficiently.

Traceability at Your Fingertips: Once a product is selected, the platform can likely display a detailed history of its journey through the supply chain. This includes information about its origin, processing, transportation, and any relevant certifications or inspections.

Empowering Transparency: By making traceability information easily accessible, the platform empowers businesses to ensure the quality and safety of their products. It also allows them to identify potential bottlenecks or disruptions in the supply chain.

Blockchain technology is revolutionizing supply chain management, offering unprecedented transparency and traceability. While the underlying technology may seem complex, platforms like IBM Food Trust and the IBM Supply Chain Intelligence Suite simplify the process, making it accessible and valuable for businesses of all sizes

CONCLUSIONS

This research highlights the transformative potential of blockchain technology in enhancing food safety and transparency within the supply chain. By offering immutable and decentralized record-keeping, blockchain ensures traceability from farm to fork. Initiatives such as IBM Food Trust validate blockchain's real-world applicability, demonstrating its ability to simplify processes, reduce fraud, and empower consumers with detailed, trustworthy information.

Blockchain's unique capacity to create tamper-proof ledgers aligns seamlessly with the needs of the food industry, revolutionizing safety protocols, mitigating counterfeit risks, and enabling swift responses to contamination issues.

However, blockchain is not a universal solution. Challenges such as scalability, interoperability, and evolving regulatory frameworks must be addressed. Successful adoption will require collaboration across all industry stakeholders to establish standards and overcome these barriers.

Despite these hurdles, blockchain holds huge promise for advancing food safety, transparency, and sustainability. Its ongoing evolution will pave the way for innovative applications that redefine global supply chains.

In conclusion, blockchain technology presents a pivotal opportunity to build safer, more transparent, and resilient food systems, fostering trust and security for future generations.

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NEW CHOCOLATE FORMULATIONS WITH IMPROVED FUNCTIONALITY BY USING CAROB AND ROSEHIP POWDERS AS PARTIAL COCOA SUBSTITUTES

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Abstract

In this study, the bioactive potential of unconventional materials, such as carob powder (CP) and rosehip powder (RP), was exploited as partial substitutes for cocoa to design new chocolate formulations. Nine formulations were prepared under laboratory conditions by substituting cocoa (w/w), as follows: 0% (control sample), 10% CP, 20% CP, 30% CP, 40% CP, 30% CP and 10% RP, 20% CP and 20% RP, 10% CP and 30% RP, respectively, 40% RP. Changes in the proximate composition and bioactive profile of chocolate were assessed based on total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity. Progressive increase in the level of CP led to improvements in the bioactive properties. The addition of CP and RP mixture resulted in a more pronounced boost in bioactive attributes with increasing RP level. The highest bioactive profile was achieved for the 40% RP formula. High levels of TPC and TFC strongly contributed to the improvement of chocolate's antioxidant activity. These findings recommend fortifying chocolate with phenolic compounds provided by CP and RP to extend the range of functional confectionary products.

Key words*: antioxidant activity, bioactive compounds, carob and rosehip powder, chocolate formulations.*

INTRODUCTION

In contemporary food technology, there is a growing emphasis on the development of functional foods that promote health and well-being. A key challenge in this area is formulating innovative chocolate products that enhance nutritional value while remaining economically viable for consumers (Salem & Fahad, 2012).

The increasing demand for food products with superior sensory and nutritional attributes has led to the investigation of novel ingredients. Food fortification serves two primary purposes: replenishing nutrients lost during processing and incorporating bioactive compounds to improve the functional properties of food. In particular, the addition of dried fruits as a source of antioxidants has been shown to enhance both the nutritional quality and health benefits of chocolate (Cagind & Ege, 2009).

Pseudo-fruits from the *Rosa* species, especially *Rosa canina* L., have long been utilized in both food and medicine due to their abundance of bioactive compounds, including vitamin C, phenolics, tannins, tocopherol, lycopene, lutein, zeaxanthin, and other carotenoids (Avallone et al., 1997; Turkben et al., 2010). Extensive research highlights the significant role of rosehip as a rich source of ascorbic acid and phenolic compounds. The ascorbic acid content in rosehip varies between 140 and 1100 mg/100 g, with *Rosa canina* L. exhibiting a comparatively lower concentration of 510 mg/100 g (Cui et al., 2014; Roman et al., 2013).

Both *in vivo* and *in vitro* studies have confirmed the anti-inflammatory and antioxidant properties of rosehip, attributed to its polyphenol, vitamin C, E and B content, as well as carotenoids, which may act synergistically (Papuc et al., 2023; Predescu et al., 2016). Furthermore, rosehip has demonstrated antimicrobial activity and has been associated with beneficial effects on chronic conditions

such as osteoarthritis and rheumatoid arthritis. Additionally, rosehip exhibits anti-diabetic and anticancer properties (Marmol et al., 2017).

In light of its well-documented bioactivity, rosehips qualify as a nutritious food and potentional functional ingredient with significant health benefits (Negrean et al., 2024; Stryjecka et al., 2025).

Carob (*Ceratonia siliqua* L.) is a valuable plant source of natural sugars, with a high sucrose content (over 50%), together with dietary fiber, protein and essential minerals including calcium, potassium and phosphorus (Ozcan et al., 2007). It also contains significant levels of various polyphenolic compounds, including anthocyanidins, hydrolysable tannins, condensed tannins and flavan-3,4-diols (Petit & Pinilla, 1995).

As reported by Papagiannopoulos et al. (2004), carob pods contain extractable polyphenols amounting to 448 mg/kg, which include gallic acid (174 mg/kg), hydrolysable tannins (26 mg/kg), condensed tannins (15 mg/kg) and derivatives of myricetin (171 mg/kg), quercetin (53 mg/kg) and kaempferol (9 mg/kg). Furthermore, carob pulp provides a moderate protein content in the range of 3-4%, while maintaining a low fat concentration of about 0.6% (Avollone et al., 1997).

Widely recognized as a natural sweetener, carob possesses a flavor and color reminiscent of cocoa and is frequently used as a cocoa substitute in a variety of food products, including chocolate, confectionery, ice cream, beverages, and baked goods (Yousif & Alghzawi, 2000). Its cocoa-like aroma arises from the thermal degradation of sugars and the formation of odorant compounds through the Maillard reaction (Fadel et al., 2006). Carob powder, produced by roasting and grinding carob pods, is characterized by a low fat content (approximately 2.5%) and is an unconventional gluten-free material of vegetable origin suitable for the food industry.

In recent decades, due to their high nutritional value, carob and rosehip powders have attracted considerable research interest as functional food ingredients (Salem & Fahad, 2012). The partial substitution of cocoa with alternative ingredients in chocolate formulations offers advantages such as reduced fat content and the introduction of unique sensory and functional properties to the final product (Fadel et al., 2006).

Our preliminary results revealed the potential of carob and rosehip powders to improve chocolate quality (Moigradean et al., 2022). Based on these findings, the aim of this study was to develop chocolate formulations with high nutritional value and improved functionality by partially replacing cocoa powder with carob and rosehip powder, as well as with mixtures of carob and rosehip powder to enhance the content of high value bioactive phenolic compounds and antioxidant properties of the designed formulations.

MATERIALS AND METHODS

Raw materials

The ingredients involved in the production recipes such as refined sugar (Pfeifer & Langen GmbH & Co. KG, Koln, Germany), milk powder (Bartex SP. Z o.o., ul. Dworcow 4A, Paslek, Poland), butter with 80% fat (Friesland Campina Romania SA, Cluj-Napoca, Romania), cocoa powder (Barry Callebaut Cocoa AG, Zurich, Switzerland), carob powder (Sano Vita SRL, Vâlcea, Romania), rosehip powder (BioPlanet SA, Leszno, Poland), were purchased from local supermarkets and local specialty stores.

Chocolate production on a laboratory scale

The chocolate was prepared according to a traditional manufacture procedure applied on a small-scale level. The materials included in the recipe of chocolate formulations were reported in Table 1. It can be seen that in the control sample, 100 g of cocoa was used, and in the other formulations, cocoa powder was substituted with carob powder (CP), rosehip powder (RP) or mixture of CP and RP, respectively. The chocolate was prepared by mixing 500 g of sugar and 150 mL of water in a pot on the stove and bring the mixture to a boil until a drop taken between the fingers forms thin threads when separated. The boiling time varies depending on the heat intensity, but typically takes about 1-2 minutes from the moment boiling starts. Once this stage is reached, we removed the pot from the heat.

Table 1. Ingredients for preparing chocolate using carob powder and rosehip powder as partial cocoa substitutes

Ingredients	C	10CP	20CP	30CP	40CP	30CP10RP	20CP20RP	10CP30RP	40RP
Sugar (g)	500	500	500	500	500	500	500	500	500
Butter 80% fat (g)	100	100	100	100	100	100	100	100	100
Milk powder (g)	250	250	250	250	250	250	250	250	250
Cocoa (g)	100	90	80	70	60	60	60	60	60
Carob powder (g)	-	10	20	30	40	30	20	10	-
Rosehips powder (g)	-	-	-	-	-	10	20	30	40
Water (mL)	150	150	150	150	150	150	150	150	150

Next, the butter (100 g) was incorporated and allowed the mixture to cool slightly.

In a separate bowl, into which have previously weighed 250 g milk powder and 100 g cocoa powder, or cocoa powder substituted with CP and RP as given in Table 1, previously sifted through a fine sieve to ensure a uniform texture, add the butter and syrup mixture and mix vigorously until smooth.

The resulting mixtures are poured into silicone moulds and left to cool to room temperature, then refrigerated for about 4 hours.

The resulting products, shown in Figure 1, were labelled according to each formula as follows:

- C - control sample with 100% cocoa;
- 10CP - 10% of cocoa was replaced with CP;
- 20CP - 20% of cocoa was replaced with CP;
- 30CP - 30% of cocoa was replaced with CP;
- 40CP - 40% of cocoa was replaced with CP;
- 30CP10RP - 30% of cocoa powder was replaced with CP and 10% with RP;
- 20CP20RP - 20% of cocoa was replaced with CP and 20% with RP;
- 10CP30RP - 10% of cocoa was replaced with CP and 30% with RP;
- 40RP - 40% of cocoa was replaced with RP.

After being taken out of the refrigerator, the chocolates were removed from the moulds, and samples were taken from each formula, packed in polypropylene food storage bags, sealed and stored at −20°C until chemical analysis.

Proximate composition and energy value evaluation

The proximate composition of chocolate formulations was evaluated in accordance with the standard method described by the Association of Official Analytical Chemists (AOAC, 2000).

The carbohydrate content (%) was calculated by subtracting the protein, ash, lipid and moisture from 100. The energy value of the chocolate was calculated according to Das et al. (2019), taking into account that 1 g of carbohydrate contributes 4 calories, 1 g of fat contributes 9 calories and 1 g of protein contributes 4 calories, as shown in equation (1).

Energy value (kcal/100 g) = carbohydrates (%) x 4 + lipids (%) x 9 + proteins (%) x 4 (1)

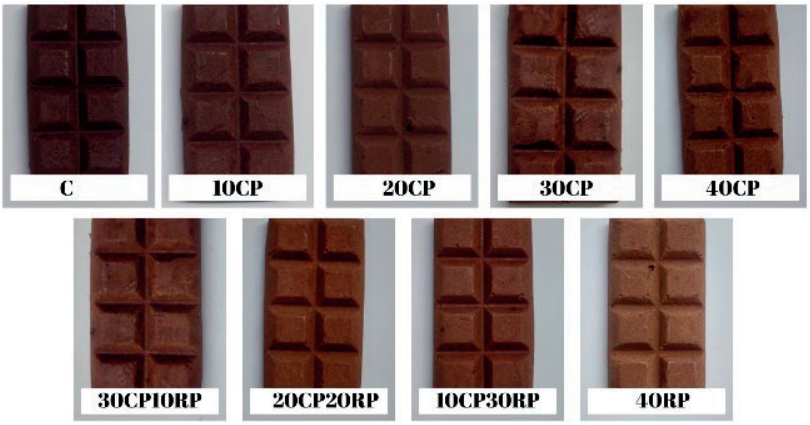


Figure 1. Chocolate formulas obtained by partially replacing cocoa powder with CP and RP (original)

Alcoholic extract preparation

The total phenolic content, total flavonoid content and antioxidant activity of the investigated samples were assessed in 70% (v/v) ethanol extracts, which were prepared according to the procedure described by Litwinek et al. (2023) and Metzner Ungureanu et al. (2020) with minor modifications. From CP, RP and defatted chocolate samples, 1.0 g was weighed into lidded containers and mixed with 10 mL of 70% (v/v) ethanol. The extraction was carried out for 2 h under continuous stirring at room temperature, and then, the mixtures were centrifuged at 10,000 rpm for 10 min (Hettich EBA 21 Centrifuge). The supernatant was collected and the residue was washed with 70% (v/v) ethanol and subjected to extraction for a further 1 h and then centrifuged in the same conditions. Afterwards, the supernatants were mixed and stored at -20°C until analysis.

Evaluation of total phenolic content (TPC)

TPC was determined by Folin-Ciocalteu method in agreement with the protocol described by Tolve et al. (2021) and Blanch et al. (2021). Dilution of the alcoholic extracts with distilled water in the following ratios 1:20 (v/v) for CP and RP and 1:10 (v/v) for chocolate samples, respectively, was carried out prior to analysis. The TPC was calculated using a standard gallic acid calibration curve in the concentration range 0.1-1.0 µM gallic acid equivalents (GAE)/mL. Results were expressed as mg GAE per 100 g of dry weight (d.s) of the sample.

Evaluation of total flavonoids content (TFC)

The total flavonoid content (TFC) was determined following the method outlined by Al-Farsi et al. (2018) with slight modifications. TFC was calculated using a standard quercetin (QE) calibration curve in the concentration range 0.5-50 µg/mL. The results were expressed as mg QE/100 g of dry weight (d.s).

Assessment of antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The free radical scavenging activity of the samples was assessed using the DPPH assay, with a 0.1 mM DPPH solution prepared in 70% (v/v) ethanol in agreement with the procedure described by Antonic et al. (2021) and Poiana et al. (2023). The previously prepared ethanolic extracts have been further diluted with 70% (v/v) ethanol in ratios of 1:200 (v/v) for CP and RP and 1:10 (v/v) for chocolate formulations, respectively. The antioxidant activity was calculated and expressed as µM Trolox equivalent (TE)/g of dry weight (d.s) of the sample.

Assessment of antioxidant activity by ferric-reducing atioxidant power (FRAP) assay

The total antioxidant potential of the samples was evaluated using the ferric reducing antioxidant power (FRAP) assay following the procedure described by Alexa et al. (2012). Dilution of the extracts with distilled water prior to analysis was 1:200 (v/v) for CP and RP and 1:10 (v/v) for chocolate formulations, respectively. The results were expressed as µM Fe^{2+} equivalent per gram of dry weight (d.s) of the sample, using a standard $FeSO_4 \cdot 7H_2O$ calibration curve in the concentration range 0.05-0.5 µM Fe^{2+} equivalents per mL.

Statistical analysis

Each determination was performed in triplicate and results were reported as mean values ± standard deviation (SD). An analysis of variance (one-way ANOVA) was carried out to assess the statistical significance of the differences between the investigated samples.

RESULTS AND DISCUSSIONS

Analytical results for the proximate composition of chocolate formulations obtained by partial replacement of cocoa powder with carob powder (CP) and rosehip powder (RP) are presented in Table 2.

It can be seen that replacing cocoa powder with RP and CP in the chocolate recipe led to an increase in the moisture content of the samples. Compared to the control sample, CP fortified chocolate formulations showed an increase in moisture content from 1.66% in the 10CP formula to 8.11% in the 40CP formula.

Table 2. Proximate composition of chocolate formulations

Sample	Protein (%)	Lipids (%)	Ash (%)	Moisture content (%)	Carbohydrates (%)	Energy value (kcal/100 g)
C	8.37 ± 0.01^{a}	16.42 ± 0.02^{a}	1.91 ± 0.03^{a}	5.42 ± 0.03^{i}	67.88	452.78
10CP	8.21 ± 0.02^{b}	16.19 ± 0.03^{b}	1.84 ± 0.01^{b}	5.51 ± 0.02^{h}	68.25	451.55
20CP	8.06 ± 0.01^{c}	16.02 ± 0.04^{c}	1.76 ± 0.01^{c}	5.62 ± 0.01^{g}	68.54	450.58
30CP	7.92 ± 0.03^{d}	15.81 ± 0.03^{d}	1.68 ± 0.02^{d}	5.75 ± 0.02^{f}	68.84	449.33
40CP	7.79 ± 0.01^{e}	15.60 ± 0.02^{e}	1.61 ± 0.01^{e}	5.86 ± 0.01^{e}	69.14	448.12
30CP10RP	7.71 ± 0.01^{e}	15.66 ± 0.04^{e}	1.66 ± 0.02^{d}	5.92 ± 0.02^{d}	69.05	447.98
20CP20RP	7.65 ± 0.02^{c}	15.73 ± 0.03^{c}	1.72 ± 0.02^{d}	6.03 ± 0.03^{c}	68.87	447.65
10CP30RP	7.58 ± 0.01^{d}	15.81 ± 0.02^{d}	1.77 ± 0.01^{c}	6.12 ± 0.02^{b}	68.72	445.09
40RP	7.49 ± 0.03^{b}	15.87 ± 0.05^{b}	1.83 ± 0.02^{b}	6.25 ± 0.04^{a}	68.56	447.03

Values are reported as the mean of three independent analyses ± standard deviation (SD). Values in a column having different superscripts are statistically different (one-way ANOVA, $p < 0.05$).

The moisture content of the RP-added chocolate samples was much higher than that of the control sample.

Replacing cocoa powder with a mixture of CP and RP powder increased the moisture content of the samples, relative to the control, by 9.22% for 30CP10RP, 11.25% for 20CP20RP and 12.91% for the 10CP30RP formulation, respectively.

In addition, replacing the cocoa powder with 40% RP resulted in the highest moisture content, resulting in an increase of 18.36% over the control sample. Therefore, a significant ($p < 0.05$) increase in the moisture content was observed in the samples with the highest amount of RP used to replace cocoa.

The data in Table 2 also shows that the protein content decreases in samples where cocoa powder was replaced by CP and RP.

The induced changes in protein content can be observed with a gradual decrease from 8.37% in control sample to 7.79% in the 40CP formula.

RP fortified samples also decrease slowly in protein content, ranging from 7.71% in 30CP10RP to 7.49% in 40RP.

The CP and RP formulations have slightly higher carbohydrate content than the control, but the differences are not significant.

The increase ranges from 0.37%, relative to the control sample in the 10CP formula, to 0.68% in the 40RP sample compared to the control, while the ash, protein and lipid contents decrease slightly.

This finding is closely related to the contribution made by CP and RP addition and has been also reported in a study on chocolate formulation with carob addition by Lagha-Benamrouche & Hezil (2023) who reported that CP enriched chocolates were slightly lower in fat and protein.

The fat content of chocolate formulations decreases slightly with substitution of cocoa powder with RP and CP.

In chocolate formulations for which cocoa was replaced by CP, the decrease in lipid content compared to the control sample ranged from 1.40% for 10CP to 4.99% for 40CP formula.

In the formulations where RP was added as a partial substitute for cocoa powder there was also a decrease in fat content compared to the control sample. However, the decrease is slightly less than in the samples where only CP was added as a cocoa replacer.

For the 30CP10RP sample a decrease of 4.62% compared to the control sample was noted, while for the 40RP sample the fat content decreased by 3.34% compared to the control sample.

The content of ash in CP chocolates was 1.91 g/100 g (control) and it very slowly decreased, to 1.61 g/100 g (40CP). In chocolates with RP, the ash content slightly increases from those with only CP, from 1.66 g/100 g in the sample 30CP10RP to 1.83 g/100 g in the 40% RP sample (40RP), but the ash content are still slightly lower than those of the C sample.

The energy value was not strongly influenced by substitution of cocoa with CP and RP decreases from 452.78 kcal/100 g in control sample, to 448.12 kcal/100 g in 40CP and to 447.03 kcal/100 g in 40RP chocolate formula.
The total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity evaluated by FRAP and DPPH assays were tested in this study for CP and RP, and the results are shown in Table 3.

Table 3. Total phenolic content and total flavonoids content along with antioxidant activity of CP and RP

Phytochemicals and antioxidant activity	CP	RP
TP (mg GAE/100 g d.s)	1686.64 ± 1.71^{b}	2307.59 ± 1.89^{a}
TF (mg QE/100 g d.s)	1108 ± 1.69^{b}	1408.28 ± 1.81^{a}
FRAP (μM Fe^{2+}/g d.s)	398.25± 1.29^{b}	517.54 ± 1.48^{a}
DPPH (μM TE/g d.s)	466.45 ± 1.37^{b}	590.54 ± 1.52^{a}

Values reported are the mean of three independent analyses ± standard deviation (SD). Values in a row having different superscripts are statistically different (one-way ANOVA, $p < 0.05$).

The most important class of bioactive substances found in CP and RP are the phenolic compounds. The antioxidant activity of rosehip is attributed to its content of polyphenols, vitamins C, E, B and carotenoids, which may exhibit synergistic effects (Marmol et al., 2017).
The data shown in Table 3 indicates that TPC of CP is 1686.64 mg GAE/100 g d.s, consistent with the findings of Ioannou et al. (2023) Information on the total phenolic content of carob pods is limited in the literature. However, it has been shown to vary depending on several factors, including the extraction method, geographical origin, variety, cultivation conditions, and maturity level (Atalanti et al., 2021).
The TPC of RP resulted was 2307.59 mg GAE/100 g d.s, similar to the findings of Igual et al. (2022) who showed results of 2482 mg GAE/100 g d.s, proving the RP has an even higher phenolic content that CP, which is consistent with the literature found in this domain.
The flavonoid content of CP (1108.0 mg QE/100 g d.s) was slightly lower than that of RP (1408.28 mg QE/100 g d.s).
In regards to the antioxidant activity of CP, a value of 398.25 μM Fe^{2+}/g d.s by FRAP assay and 466.45 μM TE/g DW by DPPH assay was obtained, while for RP, the results were 517.54 μM Fe^{2+}/g d.s by FRAP assay and 590.54 μM TE/g DW by DPPH assay.
The data in Table 3 reflect significantly ($p<0.05$) higher values of TPC, TFC, FRAP and DPPH for RP compared to CP.
The functional potential of CP and RP has been further exploited in chocolate formulations prepared according to a simple recipe commonly used at the household level. This approach integrates raw material requirements into the assessment of functional properties of traditional chocolate products.
Tests were performed to find out the changes in phytochemical content and antioxidant activity of chocolate by progressively replacing cocoa powder from 10 to 40% with CP. Then, in 40CP formulation, CP was replaced with RP from 10 to 40%.
The total phenolic content and total flavonoid content of developed chocolate formulas are shown in Table 4.

Table 4. Changes in total phenolic content and total flavonoid content of chocolate formulas

Chocolate sample	TPC (mg GAE/100 g d.s)	TFC (mg QE/100 g d.s)
C	454.32 ± 1.19^{i}	175.39 ± 1.05^{i}
10CP	471.09 ± 1.23^{h}	187.03 ± 1.1^{h}
20CP	498.25 ± 1.32^{g}	203.16 ± 1.31^{g}
30CP	526.95 ± 1.28^{f}	219.42 ± 1.15^{f}
40CP	554.53 ± 1.25^{e}	231.23 ± 1.34^{e}
30CP10RP	579.42 ± 1.34^{d}	240.49 ± 1.18^{d}
20CP20RP	597.13 ± 1.18^{c}	251.13 ± 1.22^{c}
10CP30RP	627.90 ± 1.37^{b}	263.04 ± 1.29^{b}
40RP	651.31 ± 1.41^{a}	272.8 6 ± 1.33^{a}

Values reported are the mean of three independent analyses ± standard deviation (SD). Values in a column having different superscripts are statistically different (one-way ANOVA, $p < 0.05$).

It can be seen that using CP and RP as substitutes for cocoa in different concentrations in the enriched chocolate formulations led to a progressive augmentation of TPC.
The highest value of TPC was found in the sample with 40% RP, where TPC increased by 43.35% compared to the control sample.

In the formulations of chocolate with only CP as a cocoa substitute, the increase is lower than in those with RP, but still significant, ranging from 3.69% increase from C in the 10CP sample, to 22.05% increase in the 40CP sample. The TPC increased from 454.32 mg GAE/100 g d.s in control sample to 554.53 mg GAE/100 g d.s in 40CP to 651.31 mg GAE/100 g d.s in the 40RP sample.

The total flavonoid content (TFC) also increased significantly in the samples with CP and RP. Substituting cocoa with CP increased TFC by 6.63% in the 10CP sample, to 31.83% in the 40CP sample, compared to C.

While the control sample showed TFC to be 175.39 mg QE/100 g d.s, the 10CP sample showed 187.03 mg QE/100 g d.s, and the maximum CP addition as substitute (40CP) resulted in 231.23 mg QE/100 g d.s. The highest TFC content was registered in the formulations with RP, where the TFC increase reached 55.57% in 40RP from C, more exact, a content of 272.86 mg QE/100 g d.s.

The antioxidant activity expressed by FRAP value of the chocolate formulas are shown in Figure 2.

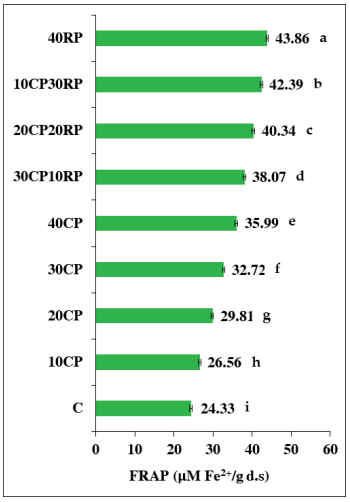


Figure 2. Changes in FRAP value of chocolate formulas. Values are the mean of three independent analyses ± SD. Values for bars with different letters are statistically different (one-way ANOVA, $p < 0.05$).

Substituting cocoa powder with CP and RP increased the antioxidant activity significantly.

Chocolate formulations that included different incorporation levels of CP and RP were found to be significantly higher ($p<0.05$) in both FRAP and DPPH values over the control.

The FRAP value increases achieved in chocolates with CP increases with 9.16% in the 10CP sample, to 47.92% in the 40CP formulation, when compared to control. The chocolates with RP the FRAP value increases significantly, from 56.47% over the C sample in the 30CP10RP formulation, to 80.27% in the formulation with 40% cocoa substituted by rosehip powder (RP).

The samples with the most RP showed the highest antioxidant activity due to the high level of bioactive compounds of RP. The FRAP value of control resulted in 24.33 μM Fe^{2+}/g d.s. The results of 40CP showed 35.99 μM Fe^{2+}/g d.s, while the highest FRAP vale was for the 40RP sample, respectively 43.86 μM Fe^{2+}/g d.s.

The antioxidant activity expressed by DPPH value of the chocolate formulas are illustrated in Figure 3.

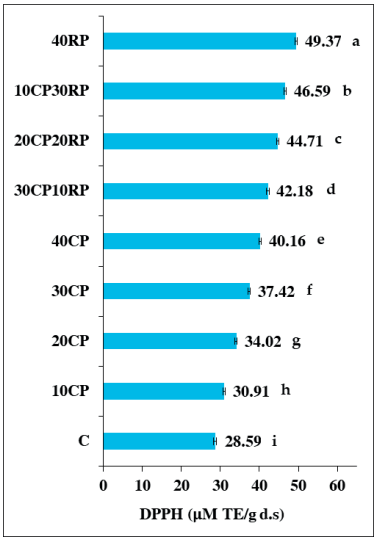


Figure 3. Changes in DPPH value of chocolate formulas. Values are the mean of three independent analyses ± SD. Values for bars with different letters are statistically different (one-way ANOVA, $p < 0.05$)

DPPH value also increases with addition of CP and RP as a cocoa substitute. Our results clearly show the significant increase of DPPH in formulations where cocoa is substituted, as follows: the 10CP sample shows an increase of 8.11% compared to control, while the 40% CP

as cocoa substitute, increases DPPH by 40.46% compared to control sample.

As with the FRAP value, the DPPH value is highest when RP is added as a substitute for cocoa, with increases from 47.53% in the 30CP10RP sample to 70.68% in the 40% RP formulation, compared to the control. Therefore, substitution of cocoa powder in chocolate manufacturing with CP and RP should improve functional properties and nutritional values of products.

CONCLUSIONS

This study demonstrates that the incorporation of CP into chocolate significantly improves the total polyphenolic compounds and total flavonoids content. In addition, PC provides a viable alternative to cocoa powder in the chocolate production.

The results show a tight correlation between the concentration of bioactive compounds and the proportion of CP added.

Chocolate samples fortified with both CP and RP showed significantly higher levels of antioxidant compounds compared to the control sample.

Due to their notable antioxidant activity, expressed both as ferric reducing antioxidant power and free radical scavenging activity, RP and CP are valuable bioactive ingredients for the development of chocolate formulations with improved functional properties.

On the basis of these findings, CP and RP are recommended as effective agents to improve the antioxidant profile and the content of bioactive compounds in chocolate.

Furthermore, the partial replacement of cocoa powder by CP and RP contributes to the diversification of chocolate varieties available on the market.

Chocolate is a complex matrix composed of several ingredients and changes in its formulation may influence the stability and preservation of antioxidants naturally present in the raw materials. The addition of CP and RP represents a practical and sustainable option for the chocolate industry.

Although RP and CP fortified chocolate shows promise as a functional food, further research is needed to fully explore its potential benefits.

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MILK`S HIDDEN TREASURE: EXPLORING WHEY PRODUCTION IN COWS, SHEEP, BUFFALO, AND GOATS

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Abstract

This study examines whey production from cow, sheep, buffalo, and goat milk, emphasizing species-specific differences in yield and nutritional composition. Drawing on recent scientific research and industry data, the analysis evaluates whey yield, protein content, and fat composition across these species. Cow milk processing emerges as the largest source of whey, while whey derived from sheep and buffalo milk exhibits higher protein concentrations (1.1-1.2%). The study provides a review of advanced whey processing technologies, including ultrafiltration, fermentation, and spray drying, as well as novel applications in functional foods, biofertilizers, and other high-value products. The findings highlight the potential of optimizing processing methodologies and implementing sustainable utilization strategies to maximize the economic and environmental value of whey. The study concludes that adopting innovative technologies and addressing key challenges such as processing costs and resource efficiency could position whey as a pivotal resource within the framework of the circular economy.

Key words*: dairy byproducts, milk, sustainability, whey.*

INTRODUCTION

Whey, a significant byproduct of cheese manufacturing, represents both an economic challenge and an environmental concern due to its substantial global production and high biochemical oxygen demand (BOD) when improperly discarded (Osorio-González et al., 2022). Globally, whey production reaches approximately 160 million tons annually, accentuating the need for sustainable disposal and valorization strategies to minimize environmental impact while maximizing economic benefits (Osorio-González et al., 2022). Whey has long been recognized for its nutritional and medicinal properties, dating back to the fifth century BC when the Greek physician Hippocrates prescribed it for gastrointestinal disorders and immune system enhancement (Guo & Wang, 2019; Smithers, 2015). Historically, whey utilization has been prominent in cheesemaking practices across different cultures. The production of traditional whey cheeses such as Ricotta in Italy dates back to Roman times, with documented practices described by Columella around 50 AD in his agricultural treatise De Re Rustica (Kindstedt, 2018; Gobbetti et al., 2018; Licitra & Carpino, 2014). Similar historical significance is noted in Norwegian whey cheeses like Gjetost and Brunost, whose origins trace back nearly a millennium to Viking traditions (Kosikowski & Mistry, 1997). In contemporary contexts, whey cheeses are produced globally under diverse regional names, reflecting distinct cultural traditions: Anthotyros and Manouri in Greece, Urda in Serbia and Romania, Lor in Turkey, Anari in Cyprus, Skuta in Croatia, and Requeijão in Portugal, among others (Fox et al., 2017; Papademas et al., 2018). Several whey cheeses have been awarded Protected Designation of Origin (PDO) status, such as Ricotta di Bufala Campana in Italy and Brocciu corse in France, emphasizing their importance in preserving regional history and cultural heritage (eAmbrosia, 2003). Beyond its cultural significance, whey is recognized for its exceptional nutritional profile and health benefits, particularly when sourced from sheep's milk. Sheep's milk whey and derived fermented products like yogurt and kefir are abundant in fatty acids, minerals (calcium, phosphorus, iron,

magnesium), vitamins (B1, B2, B5, C), and bioactive compounds (catechol, vanillin, ferulic acid, salicylic acid), which provide potent antibacterial, immunomodulatory, and antioxidant properties (Molik et al., 2008; Zhang et al., 2006; Matran et al., 2023). These bioactive compounds confer significant health benefits, including cardiovascular protection, obesity prevention, metabolic syndrome reduction, and improved insulin sensitivity, thereby helping prevent type II diabetes (Lordan et al., 2018b; Megalemou et al., 2017; Sayon-Orea et al., 2017; Gijsbers et al., 2016). Recent research has highlighted additional therapeutic potentials of whey-derived fermented beverages like kefir. Animal studies indicate kefir consumption significantly reduces precancerous lesions and improves colonic antioxidant activity, potentially through the modulation of intestinal microflora and short-chain fatty acid production (dos Reis et al., 2019; Hsu et al., 2018). Moreover, kefir displays notable anticancer activities, such as inhibiting glioblastoma cancer cells (Fatahi et al., 2021), accelerating wound healing processes (Oryan et al., 2019), and enhancing physical performance while reducing lactic acid buildup post-exercise in humans (Lee et al., 2021).

Sheep's milk whey also serves as a valuable ingredient in nutraceuticals, infant formulas, prebiotics, and probiotics, offering a suitable alternative to human breast milk (Pulina et al., 2018). Thus, the comparative study of whey derived from cows, sheep, buffalo, and goats is essential to identify optimal applications for its sustainable management and valorization. By analyzing compositional and functional differences among these animal sources, targeted strategies for maximizing economic value and minimizing environmental burdens can be effectively developed.

MATERIALS AND METHODS

A systematic literature review was conducted to compile and synthesize relevant information regarding whey production, bioactive components, and technological approaches in dairy species including cows, sheep, buffalo, and goats. Following methodologies previously described by Guiné et al., 2020, and Guiné et al., 2021, the research began by selecting an appropriate topic based on current knowledge gaps identified in existing literature. The scientific databases utilized for comprehensive searches included Web of Science, Science Direct, Scopus, and PubMed. Specific key words such as "whey production", "bioactive compounds in whey", "cheese whey valorization", and "milk bioactive substances" were employed to retrieve relevant peer-reviewed publications. Articles selected spanned from 1997 to 2025, ensuring both historical perspectives and the most recent advancements were adequately represented. Particular emphasis was placed on publications from the last five years, constituting more than half of the sources reviewed. Comparative analysis methods were employed to examine compositional differences among whey obtained from the different animal species, as well as to evaluate variations in bioactive profiles and technological strategies employed in whey valorization. Information was organized into detailed tables and figures to enhance clarity and facilitate direct comparisons across the various species and bioactive components studied.

RESULTS AND DISCUSSIONS

Milk composition and yield vary significantly among dairy species, influencing whey volume, protein recovery, and bioactive potential. These variations impact both industrial applications and the nutritional properties of whey-based products.

Species-Specific and Regional Differences in whey Production

The amount of whey obtained differs depending on the animal species, with cows producing the highest volumes, followed by buffaloes, goats, and sheep. These species-specific differences in whey yield are illustrated in Figure 1.

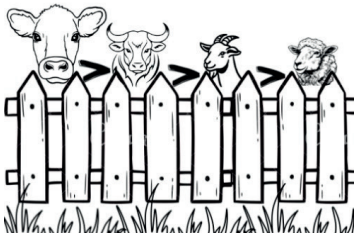


Figure 1. Species-specific whey yield (L/year). The order of animal heads illustrates the decreasing yield: Cow > Buffalo >Goat > Sheep

Cow (Bos taurus)

Cows contribute over 80% of the global milk supply, with high-yielding breeds such as Holstein-Friesians producing up to 30 kg of milk per day. Cow's milk contains 3.2–3.6% protein and 3.4–4.2% fat, making it an ideal source for whey protein isolate (WPI) and concentrate (WPC) (Bittante et al., 2022; Rashid et al., 2024). Cow whey is rich in β-lactoglobulin, α-lactalbumin, and glycomacropeptides, commonly used in sports nutrition and functional foods (Papademas, 2020; Fox & McSweeney, 1998).

Sheep (Ovis aries)

Although sheep produce less milk (0.8-1.5 kg/day), their milk is nutritionally dense, containing 5.5-6.0% protein and 6.0-7.5% fat (Flis & Molik, 2021). Sheep whey is recognized for its high bioactive peptide content, which exhibits antihypertensive, antioxidant, and immune-supporting properties (Zimecki et al., 2022). This composition makes sheep whey valuable in nutraceutical applications and high-quality cheese production (Santillo, 2009; D'Ambrosio et al., 2024).

Buffalo (Bubalus bubalis)

Buffalo milk accounts for approximately 13% of global dairy production, with major contributions from India, Pakistan, and Mediterranean regions (Arora et al., 2022). With 4.5-5.5% protein and 7.0-8.0% fat, buffalo milk generates whey with a high nutrient density (Du et al., 2019). Buffalo whey is rich in α-casein and phosphopeptides, improving calcium bioavailability and exhibiting antimicrobial effects (Talpur et al., 2007; Garau et al., 2021). Despite its potential, buffalo whey remains underutilized compared to cow whey.

Goat (Capra hircus)

Goats are well-adapted to arid regions and produce 1.0-2.5 kg/day of milk, characterized by smaller fat globules and lower αS1-casein levels, improving digestibility (Kasapidou et al., 2023). Goat whey is hypoallergenic and rich in taurine, lysozyme, and casein phosphopeptides (CPPs), making it beneficial for infant formula and medical nutrition (Pires et al., 2021; Abbas et al., 2014). It also demonstrates antimicrobial properties, increasing its potential in pharmaceutical applications (Giroux et al., 2018).

Nutritional Composition and Bioactive Compounds in Whey

The fatty acid profile of milk, especially the concentration of omega-3 polyunsaturated fatty acids and conjugated linoleic acid (CLA), can be significantly influenced through targeted dietary interventions such as oilseed supplementation. These strategies not only enhance the nutritional profile of milk fat but also contribute to the bioactive potential of whey-derived fractions, including peptides and lipid-associated compounds with health-promoting properties (Stefan et al., 2024).

The composition of whey varies significantly across species, affecting its functional and commercial applications (Barlowaska et al., 2011; Ren et al., 2015). As shown in Table 1, sheep and buffalo whey contain higher protein levels, while cow and goat whey are widely used in specialized nutritional formulations.

Table 1. Nutritional Composition of Whey from Different Species

Component (%)	Cow	Sheep	Buffalo	Goat
Yield (kg/day)	20-30	0.8-1.5	6-12	1-2.5
Protein	0.55	0.66	0.68	0.60
Fat	0.30	0.40	0.45	0.35
Lactose	4.50	4.70	4.80	4.40
Calcium	0.05	0.11	0.12	0.06
Phosphorus	0.04	0.09	0.10	0.05

According to Ben Fraj et al. (2024), whey proteins constitute up to 25% of total milk proteins in cow's milk and include key bioactive components such as β-lactoglobulin, α-lactalbumin, lactoferrin, and immunoglobulins, which are essential for immune modulation, antimicrobial defense, and nutrient transport. These findings support the increasing interest in whey fractions as functional ingredients in nutraceuticals and specialized diets.

Whey contains essential bioactive peptides with antioxidative, antimicrobial, and antihypertensive properties (Flis & Molik, 2021; Kerasioti et al., 2019). Sheep and buffalo whey exhibit higher concentrations of bioactive peptides such as lactoferrin, immunoglobulins, and casein-derived peptides (Table 2), making them promising ingredients for functional foods and medical applications (Das et al., 2022; Iram et al., 2022b).

Whey composition is influenced by genetics, dietary practices, and farm management strategies. The type of feed, particularly forage quality and supplementation, significantly affects the bioactive properties of whey (Mustonen et al., 2009; Antone et al., 2015).

Table 2. Bioactive Peptides and Functional Properties of Whey Across Species

Peptide Type	Source Species	Functional Properties
Lactoferrin	Sheep, Buffalo	Antimicrobial, Antioxidant, Immunomodulator
β-lactoglobulin peptides	Goat, Sheep	Antihypertensive, Antioxidant
α-lactalbumin peptides	Sheep, Cow	Antimicrobial, Anti-inflammatory
Casein-derived peptides	Sheep, Goat	Antidiabetic, Antioxidant, ACE-inhibitory

Recent studies emphasize that controlled feeding regimens enhance the concentration of functional peptides, optimizing the nutritional value of whey-based products (Rafiq et al., 2016; Amalfitano et al., 2024).

Recent molecular docking studies have revealed that sheep milk proteins contain bioactive peptides with promising antidiabetic and antihypertensive properties. These findings highlight the potential of sheep-derived whey proteins in the prevention and management of metabolic disorders such as type 2 diabetes and hypertension (Iram et al., 2022a).

Technological and Sustainable Approaches for Whey Valorization

Innovative technologies play a central role in the sustainable exploitation of whey, aligning with circular economy principles. Among these, membrane technologies such as ultrafiltration, microfiltration, and reverse osmosis have proven effective for concentrating whey proteins and improving cheesemaking efficiency (Argenta et al., 2021). Ultrafiltration, in particular, has become a key process for increasing protein yield and reducing waste in dairy production (Table 3).

Non-thermal microbial control methods, such as ultraviolet (UV) and near-UV light treatments, are gaining interest for extending the shelf-life of whey-derived products while preserving functional properties (Ricciardi et al., 2020a). Similarly, edible biofilms enriched with antimicrobial essential oils have emerged as sustainable packaging alternatives, enhancing product preservation and reducing environmental impact (Pires et al., 2024).

Beyond processing innovations, whey valorization has expanded into biofuel production (bioethanol, biodiesel, biohydrogen) and integrated biorefinery models, significantly reducing environmental burden and adding economic value (Carota et al., 2017; Musina et al., 2019).

Recent studies have emphasized additional value-added applications of whey. For instance, lactose-rich whey can be subjected to microbial fermentation for the production of lactic acid, a compound used in both the food and biodegradable plastics industries (Rao et al., 2024).

Table 3. Advanced technological approaches for sustainable whey valorization

Technology	Description	Application Benefits
Ultrafiltration	Protein concentration via membranes	Increased protein yield, reduced waste
UV and near-UV	Non-thermal microbial inactivation	Extended shelf-life, reduced spoilage
Biofilm packaging	Edible films with antimicrobial properties	Sustainable packaging, enhanced preservation

Furthermore, spray-drying techniques allow for the conversion of whey into highly stable powder formulations, extending shelf-life and facilitating transportation. These powders are widely used in infant formulas, sports nutrition, and bakery products. Beyond food, whey-derived proteins and peptides are increasingly incorporated into dermatological and cosmetic formulations due to their moisturizing and anti-inflammatory properties (Rao et al., 2024).

Whey has also been successfully incorporated into various food categories, including dairy, bakery, and beverages, due to its excellent emulsifying, foaming, and nutritional characteristics (Le Tien et al., 2001; Boelsma & Kloek, 2009).

Despite these advancements, challenges remain. Cost-effectiveness, scalability, and environmental sustainability continue to be major concerns in industrial applications (Smet et al., 2008; Najgebauer-Lejko & Sady, 2015). However, the

ongoing development of high-pressure technologies, enzymatic hydrolysis, and the use of whey in encapsulating probiotic cultures demonstrates the expanding utility of whey in both food and pharmaceutical sectors (Masotti et al., 2017; Rama et al., 2019).

Whey cheeses, such as Ricotta, are increasingly produced through heat- or acid-coagulation of whey, particularly from cow, goat, or sheep sources. These traditional cheeses offer a practical and profitable solution for whey valorization, especially when produced under PDO or artisanal labels (Papademas & Kotsaki, 2019; Kotoupas et al., 2007; Bintsis & Papademas, 2018).

Overall, the sustainable exploitation of whey continues to evolve, integrating technological innovation with environmental and economic priorities, confirming its role as a valuable resource rather than a waste byproduct.

Whey is extensively used in various commercial sectors including nutrition, pharmaceuticals, and functional foods (Table 4).

One of the most prominent applications is in the sports nutrition industry, where whey protein isolates (WPI) and whey protein concentrates (WPC) derived primarily from cow's milk are used due to their high biological value and rapid digestibility (Smithers, 2008; Fox & McSweeney, 2017). These proteins are rich in branched-chain amino acids (BCAAs), particularly leucine, which is critical for muscle synthesis post-exercise.

In addition to bovine sources, interest in alternative whey sources, such as sheep and goat whey, is growing due to their higher levels of bioactive peptides and better digestibility. Sheep whey contains significant amounts of lactoferrin, immunoglobulins, and oligosaccharides, which contribute to its use in infant formulas and immune-enhancing supplements (Flis & Molik, 2021; Iram et al., 2022b).

Probiotic whey beverages represent another emerging category. Products like kefir from sheep/goat whey (Tirloni et al., 2021) have shown potential to improve gut health, reduce inflammation, and deliver antioxidant compounds. Several small-scale dairy processors in Europe and New Zealand have introduced bottled kefir drinks using fermented whey, often enriched with fruits or functional herbs (Bintsis & Papademas, 2023).

Moreover, ricotta and other whey cheeses remain staple commercial products, particularly in Mediterranean countries. Ricotta di Bufala Campana (PDO), Brocciu corse (PDO), and Anthotyros are widely distributed in EU markets. Artisanal cheese-makers and dairy cooperatives have also expanded into the production of whey-based spreads, desserts, and yogurts, often with extended shelf-life through technologies like vacuum packaging or UV treatments (Bintsis & Papademas, 2023; Ricciardi et al., 2020b).

Finally, functional ingredients derived from whey, such as hydrolyzed peptides, bioactive-enriched WPC, and nanoencapsulated components, are increasingly used in medical nutrition and specialized food formulations, including diabetic-friendly snacks, immune supplements, and geriatric nutrition (Tong et al., 2000; Lordan et al., 2018a; Gupta et al., 2024).

Table 4. Examples of Commercial Applications of Whey and Bioactive Peptides

Application Type	Product Examples	Source Species	Functional Benefit
Sports nutrition	WPI, WPC powders	Cow	Muscle recovery, protein supplementation
Infant formula	Whey-based fortified formula	Sheep, Goat	Digestibility, immune support
Probiotic beverages	Whey kefir, whey smoothies	Sheep, Goat	Gut microbiota modulation, antioxydant effects
Cheese and dairy spreads	Ricotta, Anthotyros, Brocciu	Sheep, BuffaloCow	Whey valorization, traditional value
Functional supplements	Hydrolyzed peptide capsules, CLA-fortified drinks	Sheep, Cow, Goat	Anti-inflammatory, cardiovascular protection

CONCLUSIONS

Whey, as a multifaceted byproduct of cheese manufacturing, holds substantial nutritional, technological, and economic potential. Across species, sheep and buffalo whey exhibit superior protein and bioactive compound profiles, offering enhanced functional benefits compared to cow and goat whey. Innovations in membrane technologies, biorefinery integration, and biofilm packaging have significantly improved

the sustainability and efficiency of whey processing.

Commercially, whey has moved far beyond being a waste product. It is now central to several food and pharmaceutical applications, from sports nutrition to infant formula, probiotic beverages, and traditional cheeses. The presence of potent bioactive peptides in sheep and goat whey highlights the importance of diversifying beyond cow-derived ingredients. However, widespread industrial adoption remains limited by challenges such as cost-effectiveness, consumer acceptance, and regulatory barriers.

Future research should focus on optimizing extraction of specific bioactive compounds, expanding the applications of non-bovine whey, and improving the scalability of green processing technologies. Ultimately, through interdisciplinary collaboration and investment in sustainable dairy innovation, whey can be transformed into a high-value, multifunctional resource that contributes to both health and environmental goals.

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KINETICS OF NUTRITIONAL DEGRADATION OF APPLE JUICE INFLUENCED BY STORAGE CONDITIONS

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Abstract

The stability of nutrients in apple juice during storage is critical for maintaining its nutritional quality and shelf life. This study investigated the kinetics of vitamin C (ascorbic acid) and polyphenol degradation in pasteurized apple juice under varying storage temperatures (4°C, 25°C, and 35°C) over 60 days. Degradation followed first-order reaction kinetics, with rate constants (k) determined experimentally. The Arrhenius equation was applied to model the temperature dependence of degradation, revealing activation energies (E_a) for vitamin C and for polyphenols, indicating higher thermal sensitivity of vitamin C. A Q10 analysis was used in order to evaluate the vitamin C and polyphenols acceleration of degradation with time and temperature. The results highlight the importance of refrigerated storage (4°C) to minimize losses, with vitamin C retention exceeding 85% after 60 days at 4°C, compared to <40% at 35°C. These findings provide actionable insights for optimizing apple juice storage conditions and predicting shelf life using accelerated shelf-life testing (ASLT).

Key words*: apple juice, Arrhenius kinetics, nutrient degradation, pasteurization, vitamin C.*

INTRODUCTION

Apple juice is one of the most widely consumed fruit juices globally, valued not only for its pleasant taste but also for its nutritional and health-promoting properties.

It is a rich source of bioactive compounds, including polyphenols (such as flavonoids and phenolic acids), vitamin C (ascorbic acid), organic acids, and dietary fibres (Boyer & Liu, 2004; Candrawinata et al., 2012).

These constituents contribute to its antioxidant, anti-inflammatory, and potential cardioprotective effects (Hyson, 2011).

However, the nutritional quality of apple juice is highly susceptible to degradation during processing and storage, influenced by factors such as temperature, oxygen exposure, light, enzymatic activity, and packaging materials (Nicoli et al., 1999). Understanding the kinetics of nutrient degradation is crucial for optimizing storage conditions, ensuring product safety, and extending shelf life while preserving its health benefits.

Nutritional Composition and Health Benefits of Apple Juice

Apple juice contains a diverse array of phytochemicals, with chlorogenic acid, epicatechin, being among the most prominent polyphenols (Kahle et al., 2005).

These compounds exhibit strong free radical-scavenging activity, which helps mitigate oxidative stress *in vivo* (Eberhardt et al., 2000).

Additionally, vitamin C, though present in moderate amounts compared to citrus juices, plays a key role in immune function and collagen synthesis (Naidu, 2003).

The presence of soluble fibres, such as pectin, further enhances its functional properties by promoting gut health and modulating lipid metabolism (Sembries et al., 2006).

Despite these benefits, the stability of these nutrients is compromised by various physicochemical and enzymatic reactions.

For instance, vitamin C is highly sensitive to heat, oxygen, and light, undergoing oxidation to dehydroascorbic acid and further irreversible degradation (Zerdin et al., 2003).

Polyphenols, particularly those with ortho-dihydroxy structures (e.g., catechins), are prone to enzymatic browning mediated by polyphenol oxidase (PPO) and non-enzymatic oxidation, leading to color changes and loss of bioactive potential (Oszmiański et al., 2007a).

Factors Influencing Nutrient Degradation and Shelf Life

The degradation kinetics of apple juice nutrients depend on multiple extrinsic and intrinsic factors:

Temperature: Elevated storage temperatures accelerate chemical and enzymatic reactions, including Maillard browning, vitamin C oxidation, and polyphenol polymerization (Rojas & Gerschenson, 2001).

Studies suggest that refrigeration (4°C) significantly slows degradation compared to room temperature (25°C) or abusive conditions (>30°C) (Patras et al., 2009).

Oxygen Exposure: Oxidation is a major cause of nutrient loss, particularly for ascorbic acid and flavonoids. Modified atmosphere packaging (MAP) and oxygen scavengers have been shown to extend shelf life by minimizing oxidative reactions (Zerdin et al., 2003).

Light Exposure: Photooxidation, especially in transparent packaging, can degrade light-sensitive compounds like flavonoids and chlorophyll derivatives (if present in cloudy juices) (Robertson, 2010). Amber or opaque packaging is recommended to mitigate this effect.

Enzymatic Activity: Residual PPO and peroxidase (POD) activity, even after pasteurization, can lead to gradual browning and phenolic degradation (Gökmen et al., 2001). High-pressure processing (HPP) and pulsed electric fields (PEF) have been explored as alternatives to thermal treatment for enzyme inactivation (Aguilar-Rosas et al., 2007; Bahaciu et al., 2015).

pH and Water Activity: Lower pH (<3.5) generally stabilizes ascorbic acid but may accelerate non-enzymatic browning in the long term (Kaanane et al., 1988).

Safety Considerations and Microbial Stability

Beyond nutrient degradation, microbial growth is a critical factor in apple juice shelf life and safety. While pasteurization (typically 85-95°C for 15-30 s) effectively eliminates vegetative pathogens (e.g., *E. coli* O157:H7, *Salmonella*), spoilage yeasts and molds can contaminate post-processing (Vantarakis et al., 2011). Additionally, heat-resistant molds (e.g., *Byssochlamys spp.*) and their mycotoxins pose a challenge, necessitating stringent hygiene and preservatives (e.g., potassium sorbate) in some formulations (Tournas et al., 2006).

Kinetic Modelling for Shelf-Life Prediction

To predict nutrient retention and product stability, kinetic models (zero-, first-, or second-order reactions) are applied based on degradation rates under varying conditions (Labuza & Riboh, 1982). For example, vitamin C loss typically follows first-order kinetics, while browning reactions may exhibit more complex behaviour (Manso et al., 2001). Arrhenius equations further correlate temperature with reaction rates, aiding in accelerated shelf-life testing (ASLT) (Corradini & Peleg, 2007).

The nutritional quality of fruit juices, including apple juice, is significantly influenced by storage conditions, which can alter their bioactive compounds, vitamins, and overall sensory properties (Nicoli et al., 1999). Apple juice is rich in polyphenols, ascorbic acid, and antioxidants, which contribute to its health benefits, such as reducing oxidative stress and inflammation (Boyer & Liu, 2004). However, these nutrients are susceptible to degradation due to factors such as temperature, light exposure, oxygen availability, and storage duration (Oszmiański et al., 2007b).

Understanding the kinetics of nutrient degradation in apple juice is essential for optimizing storage conditions and preserving its nutritional value. Previous studies have demonstrated that vitamin C degradation follows first-order kinetics, while polyphenol oxidation depends on enzymatic and non-enzymatic reactions (Zerdin et al., 2003). Additionally, Maillard reactions and browning processes can further compromise juice quality during prolonged storage (Rojas & Gerschenson, 2001).

This study investigates the kinetic behavior of key nutritional components in apple juice under different storage conditions, temperature and time variations. By modeling degradation rates,

this research aims to provide insights into shelf-life prediction of optimal storage strategies to minimize nutrient loss.

MATERIALS AND METHODS

The pasteurised apple juice sample was purchased from a retailer. The samples were kept at 4°C, 25°C and 35°C for 10, 20, 30, 40, 50 and 60 days and the level of vitamin C and total polyphenols were determined in triple.

Vitamin C determination with 2,6-dichlorophenolindophenol (DCPIP)

Titration method with 2,6-dichlorophenolindophenol (DCPIP), based on the reduction of the blue dye DCPIP by ascorbic acid to a colourless form.

A standard AA solution was prepared: 10 mg of pure ascorbic acid were dissolved in 3% metaphosphoric acid (0.1 mg/mL). 5 mL standard AA were introduced into a flask and titrated with DCPIP from a burette until a faint pink color persisted for 15 sec. The volume of DCPIP used was noted (V_{std}).

DCPIP factor (F) was calculated with the formula:

$$F = \frac{mg\ AA\ in\ standard}{Volume\ DCPIP\ (mL)} = \frac{0.5\ mg}{V_{std}}$$

Extraction of vitamin C from apple juice: 10 g apple juice were blended with 50 mL 3% metaphosphoric acid, then centrifuge (5,000 × g, 10 min). Titration: 5–10 mL filtrate was introduced into a flask, and titrated with DCPIP until faint pink endpoint. The volume used for titration was noted (V_{sample}).

The amount of vitamin C was calculated with the following formula:

$$mg\ Vitamin\ C/100\ g = \frac{V_{sample} \cdot F \cdot Total\ extract\ volume\ (mL)}{Sample\ weight\ (g)} \cdot 100$$

Determination of Total Phenolic Content (TPC) by Folin-Ciocalteu

Assay quantified spectrophotometrically (765 nm) based on the reduction of Folin-Ciocalteu reagent by phenolic compounds, producing a blue chromophore (Singleton & Rossi, 1965). Apple juice samples were diluted (1:10) with 70% ethanol. Vortex, centrifuge (8,000 × g, 10 min). 0.5 mL diluted sample + 2.5 mL Folin- Ciocalteu (diluted 1:10 with water) then incubated (5 min, dark). After that, 2 mL Na_2CO_3 (20%) were added and the samples were incubated (30 min, 25°C, dark). Absorbances were measured at 765 nm vs. blank (water instead of sample).

Calibration Curve was prepared with gallic acid standards (0-500 µg/mL). Plot absorbance vs. concentration ($R^2 \geq 0.995$).

The amount of total polyphenolic compounds was calculated with the following formula:

$$TPC\ (mg\ GAE/100\ mL) = \frac{C \cdot V_{extract} \cdot DF}{W_{sample}} \cdot 100$$

C - gallic acid equivalent (GAE) concentration (mg/mL)

DF - dilution factor

Kinetics of nutrient degradation

The Arrhenius equation was used to model the kinetics of temperature dependence of nutrient degradation (e.g., vitamin C and polyphenols).

The Arrhenius equation describes how the rate constant (k) of a chemical reaction depends on temperature (T):

$$k = A \cdot e^{-\frac{E_a}{RT}}$$

k - reaction rate constant (e.g. for vitamin C and polyphenols degradation);

A - pre-exponential factor (frequency of collisions, s^{-1});

E_a - activation energy (J/mol);

R - universal gas constant (8.314 J/mol·K);

T - absolute temperature (Kelvin, K).

Nutrient degradation often follows first-order kinetics:

$$\frac{dC}{dt} = -k \cdot C$$

$$C(t) = C_0 \cdot e^{-k \cdot T}$$

C - nutritent concentration at time, t;

C_0 - initial concentration;

k - degradation rate constant (from Arrhenius equation).

By combining the two equations, it can be predicted nutrient loss over time at different stirage/procesing temperatures.

Most nutrient degradations are first-order (log-linear loss over time).

Retention of the nutrient during storage is the amount of the final concentration reported to the initial one, in %.

$$Retention\ (\%) = \frac{C_t}{C_0} \cdot 100$$

Degradation is calculated with the formula:

$$Degradation = 100 - Retention\ (\%)$$

Experiments were conducted at multiple temperatures (4, 15, 25 and 35°C), then it was plotted ln(k) vs. 1/T (Arrhenius plot) and then E_a and A were calculated from the graph plot slope ($-E_a/R$) and intercept (lnC). The Arrhenius-derived k was then used to calculate nutrient retention (%) at any temperature.

RESULTS AND DISCUSSIONS

Kinetics in vitamin C degradation during storage of apple juice

The level of vitamin C determined in apple juice samples stored at 4°C, 15°C, 25°C and 35°C for 0 to 60 days is shown in Table 1. For calculations and dimensional analysis correctness, time is represented in minutes.

Table 1. Vitamin C amount in apple juice samples (mg/100 g product)

Vitamin C, mg/100 g				
Temperature, °C / Time (min)	4	15	25	35
0	0.900	0.900	0.900	0.900
14400	0.854	0.789	0.702	0.702
28800	0.823	0.753	0.612	0.612
43200	0.802	0.678	0.543	0.543
57600	0.771	0.621	0.476	0.476
72000	0.756	0.591	0.425	0.425
86400	0.744	0.573	0.401	0.401

In order to determine the influence of each temperature and denaturation kinetics, Arrhenius equation was used the following steps are made:

It is calculated the C/C_0 ratio (level of ascorbic acid (C) after exposure to temperature, for each period of time, reported to initial level of ascorbic acid content, C_0), then the $\ln(C/C_0)$. These data are inserted in a graphical representation of ln (C/C0) = f (time) at each temperature.

Table 2. Determination of C/C_0 and $\ln(C/C_0)$ values for apple juice samples (in order to apply the Arrhenius equation)

Temperature, °C / Time (min)	4		15		25		35	
	C/C_0	$\ln(C/C_0)$	C/C_0	$\ln(C/C_0)$	C/C_0	$\ln(C/C_0)$	C/C_0	$\ln(C/C_0)$
0	1	0	1	0	1	0	1	0
14400	0.9520	-0.0492	0.8830	-0.1244	0.7650	-0.2679	0.5890	-0.5293
28800	0.9010	-0.1043	0.7960	-0.2282	0.6240	-0.4716	0.3820	-0.9623
43200	0.8570	-0.1543	0.7120	-0.3397	0.5030	-0.6872	0.2480	-1.3943
57600	0.8100	-0.2107	0.6350	-0.4541	0.4010	-0.9138	0.1530	-1.8773
72000	0.7680	-0.2640	0.5630	-0.5745	0.3270	-1.1178	0.0950	-2.3539
86400	0.7250	-0.3216	0.4980	-0.6972	0.2640	-1.3318	0.0510	-2.9759

Next steps in the evaluation of degradation kinetics, is to represent the ln(C/C0) function of time for each temperature. The equation is generated with Trendline function of Excel programme for linear evolution of the coordinates.

Accordingly, in Arrhenius equation, the reaction rate constant, k can be determined by the line equation, k = minus equation slope. For the situation of juice storage at 4°C (Figure 1), the equation is y = -4E-06x + 0.0031, so k = 4E-06, which means, k=0.000004.

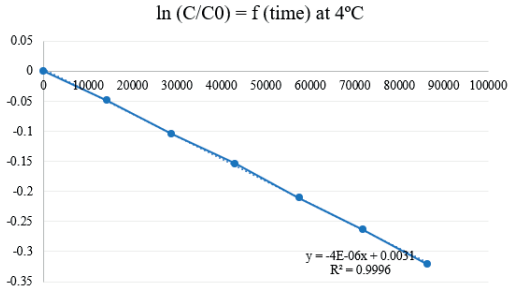


Figure 1. Representation of ln(C/C0) = f(time) at 4°C in order to determine the reaction rate

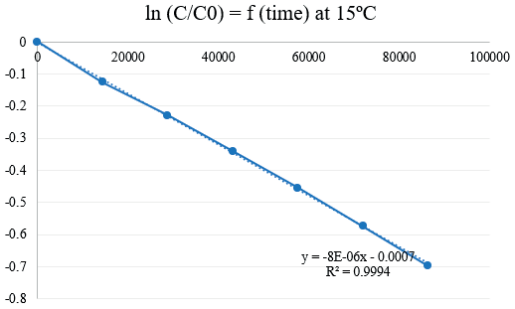


Figure 2. Representation of ln(C/C0) = f(time) at 15°C in order to determine the reaction rate

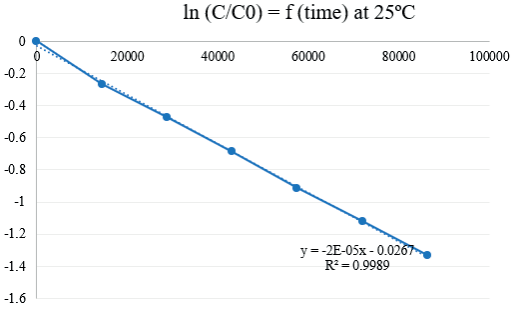


Figure 3. Representation of ln(C/C0) = f(time) at 25°C in order to determine the reaction rate

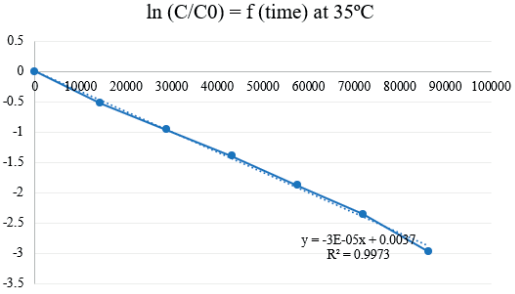


Figure 4. Representation of ln(C/C0) = f(time) at 35°C in order to determine the reaction rate

Considering the same calculation procedure, the reaction rate constant, k, were determined and the results are shown in Table 3.

The next step of the calculation is to represent the evolution of lnk function of 1/T and to identify the activation energy, Ea (Table 4).

Table 3. The reaction rate constant based on temperature

t °C	T, K	k
4	277.15	0.000004
15	288.15	0.000008
25	298.15	0.00002
35	308.15	0.00003

Table 4. Calculation of lnk and 1/T parameters

1/T	lnk
0.0036	-12.4292
0.0035	-11.7361
0.0034	-10.8198
0.0032	-10.4143

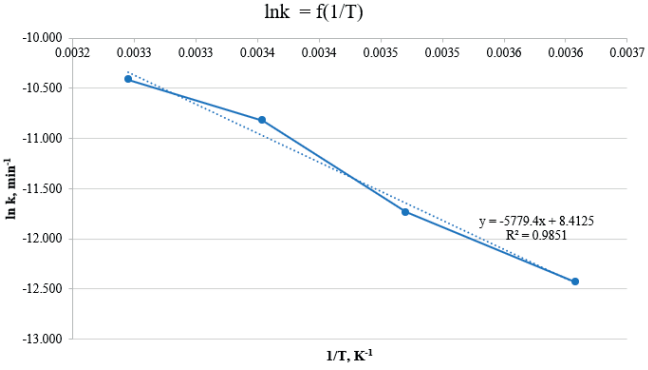


Figure 5. The evolution of the reaction rate constant (lnk) with temperature (1/T) for vitamin C degradation

From the equation shown in Figure 4 which represents the influence of temperature on reaction rate constant, the Ea/R ratio it was determined.

Table 5. Calculation of the activation energy Ea, kJ/mol for vitamin C degradation

Parameter	Unit of measure	Value	
-Ea/R	K	-5779.4	$-\frac{E_a}{R}$ → LN (A)
Ea/R	K	5779.4	
R – universal gas constant	J/mol K	8.314	
Ea	J/mol	48,049.93	
Ea – activation energy	kJ/mol	**48.05**	**y = -5779.4x + 8.4125**
LN(A)		**8.4215**	$A = e^{8.4125} = \mathbf{4503.01\ min^{-1}}$

The activation energy (E_a) represents the energy barrier that must be overcome for vitamin C degradation to occur. The measure unit is kJ/mol. Higher E_a = stronger temperature dependence (i.e., degradation accelerates more sharply as temperature rises).
In apple juice storage, the range of $E_a \approx$ 60- 65 kJ/mol (Vikram et al., 2005; Aadil et al., 2019).
High E_a: Vitamin C degradation is highly sensitive to temperature changes.
For example, a 10°C increase (e.g., from 4°C to 14°C) may double the degradation rate ($Q_{10} \approx$ 2.5).
Low E_a (<30 kJ/mol): Weak temperature dependence (e.g., some polyphenols).
Practical Impact: at 4°C (277 K): slow degradation (long shelf life), at 35°C (308 K): rapid degradation (short shelf life).
The **pre-exponential factor (A)**, also called the **frequency factor**, is a critical parameter in the Arrhenius equation that reveals fundamental insights about reaction dynamics:

$$k = A \cdot e^{-\frac{E_a}{RT}}$$

A represents the collision frequency (for gas-phase reactions) or attempt rate (for condensed phases) of molecules in the correct orientation for reaction. While Ea governs temperature sensitivity, A sets the "baseline" reaction speed when T→∞.
For complex systems (e.g., foods), in degradation reactions, A encodes molecular mobility in the matrix (e.g., water activity affects diffusion), catalytic effects (e.g., metals, enzymes), pH-dependent protonation states.

Table 6. The role of A value in practice

Application	Interpretation of A	Example
Shelf-life prediction	High A - faster degradation, even at low E_a	Vitamin C in contaminated juice degrades rapidly despite moderate E_a.
Process optimisation	Low A - longer processes time needed	Pasteurization of viscous liquids requires higher temperature to compensate for low molecular mobility (low A)
Quality control	A value shifts indicate formulation changes (e.g. antioxidants added)	Adding chelators reduces A values for oxidation reactions by sequestering metals

We calculated the A value and the results shown $A = e^{8.4125} = 4503.01\ min^{-1}$, which is **$A=6.48 \cdot 10^6\ day^{-1}$** for vitamin C degradation, which suggests: high frequency of reactive events are likely due to: high solubility of vitamin C → frequent reactant encounters, susceptibility to multiple degradation pathways (oxidation, hydrolysis).
Typical A for vitamin C: 10^5-$10^8\ day^{-1}$, our determined value falls within expected range. Deviations may reflect differences in juice matrix (e.g., polyphenols acting as pro-oxidants) (Tikekar et al., 2011).
The reaction rate constant analysis and comparison can be used in order to identify the speed of denaturation of a food product, which is directly related to shelf-life prediction.

Table 7. The rate of decreasing of vitamin C in apple juice due to storage conditions and time

t °C	k	$\frac{k_{T2}}{k_{T1}}$	Comments
4	0.000004		
15	0.000008	$\frac{k_{15}}{k_4} = \frac{0.000008}{0.000004} = 2$	Vitamin C in apple juice degrades 2 times faster at 15°C than at 4°C
25	0.00002	$\frac{k_{25}}{k_4} = \frac{0.00002}{0.000004} = 5$	Vitamin C in apple juice degrades 5 times faster at 25°C than at 4°C
35	0.00003	$\frac{k_{35}}{k_4} = \frac{0.00003}{0.000004} = 7.5$	Vitamin C in apple juice degrades 7.5 times faster at 35°C than at 4°C

Kinetics in total polyphenols degradation during storage of apple juice

The level of total polyphenols was determined in apple juice samples stored at 4°C, 15°C, 25°C and 35°C for 0 to 60 days is shown in Table 8. For calculations and dimensional analysis correctness, time is represented in minutes.

Table 8. Total polyphenols content of apple juice samples (mg GAE/100 mL)

Total polyphenols content (TPC) (mg GAE/100 mL)				
Temperature, °C / Time (min)	4	15	25	35
0	268.75	268.75	268.75	268.75
14400	264.72	258.54	249.13	229.51
28800	260.96	252.09	235.16	199.41
43200	256.93	242.68	220.38	171.46
57600	252.63	234.08	205.59	147.54
72000	248.06	224.41	188.66	124.43
86400	243.22	215.81	174.15	104.01

Table 9. Determination of C/C_0 and $\ln(C/C_0)$ values for apple juice samples (in order to apply the Arrhenius equation)

Total polyphenols content (TPC), mg GAE/100 mL								
Temperature, °C / Time (min)	4		15		25		35	
	C/C_0	$\ln(C/C_0)$	C/C_0	$\ln(C/C_0)$	C/C_0	$\ln(C/C_0)$	C/C_0	$\ln(C/C_0)$
0	1	0	1	0	1	0	1	0
14400	0.9850	-0.0151	0.9620	-0.0387	0.9270	-0.0758	0.8540	-0.1578
28800	0.9710	-0.0294	0.9380	-0.0640	0.8750	-0.1335	0.7420	-0.2984
43200	0.9560	-0.0450	0.9030	-0.1020	0.8200	-0.1985	0.6380	-0.4494
57600	0.9400	-0.0619	0.8710	-0.1381	0.7650	-0.2679	0.5490	-0.5997
72000	0.9230	-0.0801	0.8350	-0.1803	0.7020	-0.3538	0.4630	-0.7700
86400	0.9050	-0.0998	0.8030	-0.2194	0.6480	-0.4339	0.3870	-0.9493

In order to determine the influence of each temperature and denaturation kinetics, Arrhenius equation was used the following steps are made:

It is calculated the C/C_0 ratio (level of total polyphenols TPC (C) after exposure to temperature, for each period of time, reported to initial level of TPC (C_0), then the $\ln(C/C_0)$.

These data are inserted in a graphical representtation of ln (C/C0) = f (time) at each temperature.

Next steps in the evaluation of degradation kinetics, is to represent the ln(C/C0) function of time for each temperature. The equation is generated with Trendline function of Excel programme for linear evolution of the coordinates.

Accordingly, in Arrhenius equation, the reaction rate constant, k can be determined by the line equation, k = minus equation slope. For the situation of juice storage at 4°C (Figure 1), the equation is y = -1E-06x - 0.0166, so k = 1E-06, which means, k=0.000001

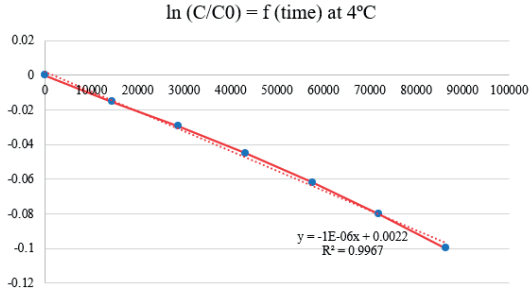


Figure 6. Representation of ln(C/C0) = f(time) at 4°C in order to determine the reaction rate

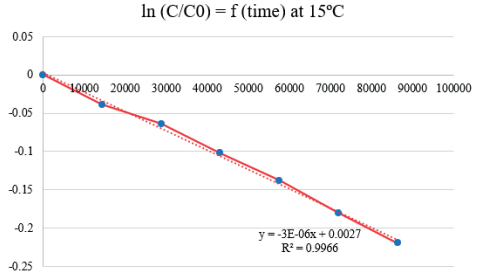


Figure 7. Representation of ln(C/C0) = f(time) at 15°C in order to determine the reaction rate

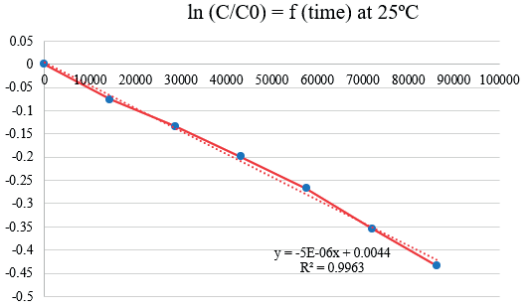


Figure 8. Representation of ln(C/C0) = f(time) at 25°C in order to determine the reaction rate

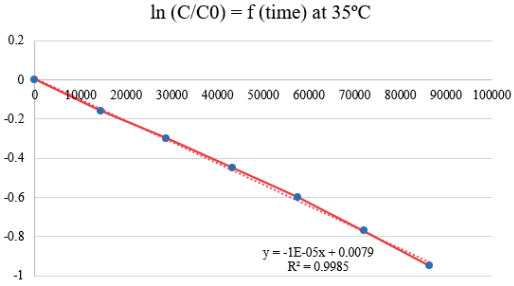


Figure 9. Representation of ln(C/C0) = f(time) at 35°C in order to determine the reaction rate

The reaction rate constant, k, were determined and the results are shown in Table 10.
The next step of the calculation is to represent the evolution of lnk function of 1/T and to identify the activation energy, Ea (Table 11).

Table 10. The reaction rate constant based on temperature

t °C	T, K	k
4	277.15	0.000001
15	288.15	0.000003
25	298.15	0.000005
35	308.15	0.00001

Table 11. Calculation of lnk and 1/T parameters

t °C	T, K	1/T	k	lnk
4	277.15	0.0036	0.000001	-13.815511
15	288.15	0.0035	0.000003	-12.716898
25	298.15	0.0034	0/000005	-12.206073
35	308.15	0.0032	0.00001	-11.512925

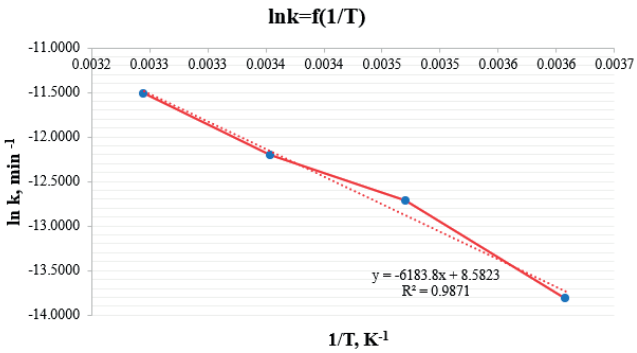


Figure 10. The evolution of the reaction rate constant (lnk) with temperature (1/T) for TPC degradation

From the equation shown in Figure 10 which represents the influence of temperature on reaction rate constant, the Ea/R ratio it was determined.

Table 12. Calculation of the activation energy Ea, kJ/mol, for TPC degradation

Parameter	Unit of measure	Value	Formula
$-E_a/R$	-6183.8	K	
E_a/R	6183.8		
R	8.314	**J/mol K**	
E_a	**51,412.11**	**J/mol**	
E_a	**51.41**	**kJ/mol**	

We calculated the A value and the results shown $A = e^{8.4125} = 5{,}336.37\ min^{-1}$, which is **$A=7.68 \cdot 10^6\ day^{-1}$** for total polyphenol degradation).

The pre-exponential factor (A) for polyphenol degradation in apple juice typically falls within the range of 10^4–10^7 day^{-1} (depending on the specific polyphenol class and storage and processing conditions. Our values are falling into this area (Krapfenbauer et al., 2007).

The reaction rate constant analysis and comparison can be used in order to identify the speed of denaturation of a food product, which is directly related to shelf life prediction.

Table 13. The rate of decreasing of total polyphenols (TPC) in apple juice due to storage conditions and time

t °C	k	$\frac{k_{T2}}{k_{T1}}$	Comments
4	0.000001		
15	0.000003	$\frac{k_{15}}{k_4} = \frac{0.000003}{0.000001} = 3$	TPC in apple juice degrades 3 times faster at 15°C than at 4°C
25	0/000005	$\frac{k_{25}}{k_4} = \frac{0.00005}{0.000001} = 5$	TPC in apple juice degrades 5 times faster at 25°C than at 4°C
35	0.00001	$\frac{k_{35}}{k_4} = \frac{0.0001}{0.000001} = 10$	TPC in apple juice degrades 10 times faster at 35°C than at 4°C

A high A (e.g., 10^6 day^{-1}) means polyphenols degrade rapidly even at low temperatures (e.g., quercetin in stored juice). Formulators can reduce A by adding antioxidants (e.g., ascorbic acid) or chelators (e.g., citrate).

CONCLUSIONS

This study reveals that vitamin C and polyphenols in apple juice degrade following first-order kinetics, with temperature being the dominant factor, with degradation rates increasing significantly at higher storage temperatures (4°C to 35°C).

Vitamin C shows greater thermal sensitivity (E_a=48.05 kJ/mol) than polyphenols (E_a=51.41 kJ/mol), though quercetin glycosides degrade fastest among polyphenols.

Key findings include:

- Each 10°C temperature increase accelerates degradation 2-3× (vitamin C) and 3-10× (polyphenols)
- Refrigeration (4°C) preserves >85% vitamin C and >90% polyphenols for 60 days versus <60% retention at 35°C
- Mild pasteurization (85°C, ≤30s) optimally balances microbial safety with nutrient preservation.

The validated Arrhenius models (R^2>0.99) enable precise shelf-life predictions, while oxygen-barrier packaging and potential non-thermal methods offer practical preservation solutions.

These findings provide actionable guidance for maintaining nutritional quality in apple juice production and storage.

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BEETROOT POMACE POWDER AS A BIOACTIVE POWDER INGREDIENT IN MAYONNAISE FORMULATION

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Abstract

Beetroot pomace powder (BPP), a by-product of beetroot processing, is a rich source of bioactive compounds, including phenolics and betalains These compounds provide antioxidant, anti-diabetic, anti-inflammatory, and antiproliferative activities. The integration of beetroot pomace powder (BPP) as a bioactive component in mayonnaise formulation signifies an innovative method for improving mayonnaise's nutritional and functional attributes. This research examines the impact of BPP incorporation (with varying BPP concentrations) on the physicochemical characteristics, phytochemical, color, sensory qualities, and texture of BPP-enriched mayonnaise. The findings indicated that BPP markedly improved the antioxidant activity (7.66-9.75 µmol TE/g dw) and betalain contents (2.18-3.93 mg/g) of mayonnaise while maintaining sensory acceptability, with an optimal 3% BPP inclusion level. Moreover, BPP enhanced mayonnaise's aesthetic properties. The results indicate that beetroot pomace is a feasible natural coloring ingredient, consistent with consumer demand for healthier and more sustainable food products. Subsequent investigations may examine the wider applicability of BPP in food systems and its long-term storage effects.

Key words*: agro-industrial by-products, antioxidants, betalains, phytochemicals, value-added food products.*

INTRODUCTION

The rising consumer desire for functional foods that integrate nutritional advantages with improved health benefits has propelled the investigation of novel food ingredients. Agro-industrial by-products, including beetroot pomace, have become attractive resources due to their abundant bioactive components (Bharat Helkar, & Sahoo, 2016; Simioniuc et al., 2021). Approximately 15-30% of the raw material generated by the juice company, comprising red beetroot peel and pulp, is regarded as waste and is subsequently discarded as animal feed. Beetroot pomace, the fibrous residue remaining post-juice extraction, is a nutrient-dense by-product that has attracted interest for its possible uses in food systems. Often discarded or utilized as animal feed, beetroot pomace encompasses a variety of beneficial substances, including betalains, polyphenols, dietary fiber, and vital minerals such as potassium, magnesium, and calcium. These substances not only improve the nutritional quality of food products but also augment many functional and technological attributes (Sahni & Shere, 2017).

Beetroot pomace is distinguished by its elevated betalain concentration, consisting of betacyanins (red-violet pigments) and betaxanthins (yellow pigments). These chemicals are powerful antioxidants with documented anti-inflammatory, anticancer, and cardiovascular protective properties. The phenolic components in beetroot pomace augment its antioxidant ability, rendering it a valuable element for health-oriented food compositions. Additionally, the dietary fiber content promotes gastrointestinal health and aids in the regulation of blood glucose and cholesterol levels, rendering beetroot pomace a functional food component with extensive health advantages (Vulić et al., 2014).

Betalains, potent antioxidants, are present in beetroot by-products, accounting for their vibrant red and yellow hues. Delgado-Vargas et al. (2000) indicated that betacyanins comprise around 75-95% of the pigments present in beetroot, whereas betaxanthins constitute the remaining 5-25%.

Betalains have been demonstrated to have antiviral, antioxidant, antibacterial, antifungal, antidiabetic, anticancer properties, anti-lipidemic effects, cardiovascular effects, and anti-inflammatory effects (Sadowska-Bartosz & Bartosz, 2021).

Commercially available red beet-based colorants exist as either juice concentrates (derived by vacuum concentration of red beet juice to 60-65% total solids) or powders, with a pigment level ranging from 0.3% to 1%. In 2009, betalains from red beetroot comprised 10% of the worldwide food coloring market (Manchali et al., 2012).

The application of red beetroot by-product powders in various food products has garnered attention due to its capacity to improve nutritional value and provide functional benefits. Kumar et al. (2018) explored the development of ginger (*Zingiber officinale*) candy enriched with beetroot pomace extract to enhance its phytochemical profile and functional properties. The study determined that the maximum phytochemical activity was achieved under optimized conditions, specifically with a blanching time of 7.81 minutes and the addition of 9.24% beetroot pomace extract. Lazar et al. (2022) obtained a value-added mayonnaise with different concentrations of beetroot peels. The findings provide a new formulation and technological understanding of the impact of beetroot peel-powder enrichment on the physical, sensory, and textural attributes of mayonnaise.

Mayonnaise, a popular sauce, serves as a suitable medium for incorporating bioactive compounds due to its emulsion structure and market approval. Nevertheless, the conventional composition of mayonnaise frequently lacks substantial health-enhancing qualities. The oxidation of mayonnaise can be inhibited with the incorporation of natural antioxidants derived from plant extracts (Khalid et al., 2021). Fortifying mayonnaise with beetroot pomace powder (BPP) has the potential to improve its nutritional profile while maintaining its organoleptic characteristics, including texture, flavour, and colour. Moreover, integrating BPP adheres to the tenets of sustainability and circular bioeconomy by minimizing food waste and enhancing the valuation of by-products.

This study examines the viability of BPP as a bioactive component in mayonnaise formulation. The study examines the powder's impact on the physicochemical qualities, bioactives, antioxidant activity, colour, texture, and sensory attributes of the red BPP-supplemented product. The results may facilitate wider utilization of beetroot pomace in various food systems, offering creative solutions for health-conscious customers and the food sector.

MATERIALS AND METHODS

Beetroot (*Beta vulgaris* var. 'Rubiniu') was procured from a local farmer in Iasi. All ingredients utilized for the mayonnaise were procured from a local market, comprising sunflower oil, lemon juice, egg powder, vinegar, and salt.

All the following chemicals were obtained from Sigma-Aldrich (Steinheim, Germany): ethanol (70%), citric acid, methanol, 2,2-diphenyl1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, sodium nitrite solution, sodium acetate solution, sodium hydroxide, aluminium chloride, potassium chloride, sodium carbonate.

Beetroot Pomace Powder Preparation

The red beetroot of the 'Rubiniu' variety was purchased from a local producer in Iasi County, Romania. The beetroots were washed, after which the skins were removed. Next, the raw materials were pre-shredded, and then subjected to compression. The beetroot juice was extracted with a Bosch MES3500 (Philips Consumer Lifestyle B.V., Drachten, The Netherlands) equipment, resulting in the red beetroot pomace. The beetroot pomace underwent freeze-drying for 50 hours at –45°C under a pressure of 0.10 mBar till achieving a moisture content of 9.00%, utilizing a freeze-dryer (Biobase bk-fd10t equipment, Jinan, China). Finally, the dried pomace was then ground with a grinder and stored in a hermetically sealed container in the dark at room temperature.

Ultrasound-Assisted Extraction

For the phytochemical characterization of red beetroot pomace extract, the extraction of biologically active compounds from beetroot pomace was carried out by homogenizing 1 g of powder obtained previously with 9 mL of ethanol (70%) and 1 mL of citric acid (1%). The obtained mixture was maintained on the ultrasonic bath (Elmasonic S 180 H, Elma, Germany) for 40 min, at a temperature of 30±2°C and 40 kHz. Then the beetroot extract was centrifuged for 10 min at 5500 rpm, 8°C, and the supernatant was collected and characterized for phytochemical content.

Determination of total betalain content

The betaxanthins and betacyanins in the extracts were measured using UV-Vis spectrophotometer (Analytik Jena-Specord 210 Plus, Germany) at 538 nm and 480 nm. (Kushwaha et al., 2018). The results were expressed as milligrams per gram of dry weight (dw). The overall betalain concentration (in mg per 100 g of sample) was ascertained by totaling the value's contents for betacyanin and betaxanthin.

Determination of total flavonoid content

The technique outlined by Dewanto et al. (2002) was employed to evaluate the total flavonoid content. A 250 µL sample is combined with 1.25 mL of purified water and 0.075 mL of 5% sodium nitrite. The mixture is allowed to react for 5 minutes, after which 0.15 mL of 10% aluminium chloride is introduced and permitted to react for an additional 6 minutes. Add 0.5 mL of 1 M sodium hydroxide and 0.775 mL of distilled water. The absorbance of the resultant mixture is promptly measured at a wavelength of 510 nm. The findings were expressed as milligrams of catechin equivalents (CE/g of dw). Quantification of results was performed based on the catechin calibration curve.

Determination of total polyphenol content

The total polyphenol content was evaluated using the Folin-Ciocalteau technique, and the concentrations were expressed in milligrams of gallic acid equivalents (GAE/g of dw) (Horincar et al., 2019). In this procedure, 0.20 mL of the sample was combined with 15.8 mL of distilled water and 1 mL of Folin-Ciocalteu reagent in a test tube. The mixture was allowed to stand for 6 minutes, after which 3 mL of 20% sodium carbonate was added. It was then left to react for one hour at room temperature in the dark. The absorbance was recorded at 765 nm.

Determination of antioxidant activity

The antioxidant activity was measured using the DPPH free radical-scavenging technique and expressed as µM Trolox Equivalents (TE/g of dw) (Horincar et al., 2019). To analyse the sample, 100 µL of it was added to a test tube containing 3.9 mL of DPPH solution. For the control, 100 µL of methanol was mixed with 3.9 mL of DPPH. After incubating the mixture in the dark for 90 minutes, the absorbance was measured at a wavelength of 515 nm using a UV-visible spectrophotometer (Analytik Jena Specord 210 Plus, Jena, Germany). Additionally, a standard curve was created using different concentrations of Trolox. The inhibition % was determined using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

Preparation of red BPP-supplemented mayonnaise samples

The recipe for mayonnaise samples contains the following ingredients: sunflower oil (80%), egg powder (8%), vinegar (2%), water (6%), lemon juice (3%), salt (0.2%), and red beetroot pomace powder (M1-1% and M2-2%).

A coarse emulsion was formed in water by dissolving egg powder, lemon juice, salt, and vinegar. Mayonnaise was obtained by incrementally incorporating the oil into the watery liquid and rapidly mixing the ingredients for 13 minutes. Then red beetroot pomace was added at different proportions (1% - M1 and 2% - M2). It was homogenized so that the composition was uniform in terms of color and texture. After preparation, the mayonnaise was kept at a temperature of 4°C to carry out the physicochemical and phytochemical analyses. For ease of comparison, a control sample (C) was also prepared using the same processing but without the inclusion of beetroot pomace.

Physicochemical, phytochemicals, and antioxidant activity evaluation of red BPP-supplemented mayonnaise

The samples were analyzed for moisture, ash, total protein, lipids, and carbohydrates using the standard procedures of AOAC and Gavril (Rațu) et al. (2024).

The methodologies previously described were employed to evaluate the total betalain, total flavonoid content, polyphenolic content, and antioxidant activity of red BPP-supplemented mayonnaise samples.

Colour evaluation

The colour characteristics of the red BPP-supplemented mayonnaise were measured using a CR300 Chroma Meter (Konica Minolta, Sensing Americas, Inc., Ramsey, NJ, USA) equipped with a D65 illuminant. The CIELAB colour parameters (L*, a* and b*) were recorded in triplicate. Hue Angle (Hue angle = arctan(b*/a*) for quadrant I (+a*, +b*), the Chroma or colour intensity (Chroma=$\sqrt{(a^*)^2 + (b^*)^2}$), and the total colour difference, ($\Delta E = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2}$), were also determined (Dag et al., 2017).

Textural parameters

Texture analysis was conducted using a Brookfield Ametek CT3 Texture Analyser, employing the Texture Profile Analysis (TPA) technique. A double dispersion technique was employed on a 38.1 mm diameter acrylic cylinder sample to attain a depth of 20 mm. The test velocity was established at one millisecond per second, the trigger load at 0.067 N, and the load cell at 9.8 N. The textural characteristics of hardness, cohesiveness, adhesiveness, and chewiness were computed utilizing the TexturePro CT V1.5 program. Each sample was replicated three times.

Sensory evaluation

A 9-point hedonic scale, ranging from 1 (dislike very much) to 9 (like very much), was utilized in the questionnaire to evaluate the experimental mayonnaise samples. The assessment focused on several attributes: appearance, color, spreadability, consistency, and taste; aroma, odor, aftertaste, and general acceptability (Raikos et al., 2016). Twenty untrained panelists were selected for sensory analysis, including students, and staff members from the Faculty of Agriculture, Iasi University of Life Sciences, Romania. Samples were served at room temperature, with water and crackers provided to cleanse the palate between tastings.

Statistical analysis

The results are presented as the mean ± standard deviation. Statistical differences among the results were assessed using analysis of variance (ANOVA). Tukey's test was employed to identify significant pairwise comparisons, utilizing Minitab 18 software. A p-value of less than 0.05 was considered indicative of statistical significance in all tests. The XLSTAT software (Paris, France, 2019, version 2024.3) was utilized for Principal Component Analysis (PCA).

RESULTS AND DISCUSSIONS

Phytochemical and color characterization of red beetroot pomace extract

Table 1 shows the phytochemical profile of the ethanolic extract obtained by the ultrasound-assisted extraction method from red beetroot pomace. It can be seen from Table 1 that the red beetroot pomace extract showed a high content of biologically active compounds, especially polyphenols (225.90±1.41 mg GAE/100 g dw).

Table 1. Phytochemical and color characteristics of beetroot pomace extract

Parameters	Beetroot pomace extract
Total betalain content (mg/100 g dw)	211.43 ±1.29
Total flavonoid content (mg CE/ 100 g dw)	112.65 ±1.17
Total polyphenol content (mg GAE/100 g dw)	225.90 ±1.41
Antioxidant activity (µMol TE/g dw)	42.11 ±0.32
DPPH inhibition (%)	79.84 ±0.49
L*	35.64±0.39
a*	30.88±0.43
b*	4.82±0.10
Hue angle	0.15±0.03
Chroma	31.25±0.25

Also, a total betalain content of 211.43±1.29 mg/100 g was noted. High concentrations of bioactive compounds led to high antioxidant activity with a DPPH free radical inhibition capacity of approximately 80%. According to Vulić et al. (2012), the betalain content in various beetroot genotypes ranged between 0.75 and 3.75 mg/g dry weight of beetroot pomace extract. Kujala et al. (2001) highlighted that the extraction process and the choice of solvent significantly influence the efficiency of phenolic extraction from beetroot by-products. They reported a polyphenol content of 24.1 mg GAE/g in a methanolic extract. Alshehry (2019)

reported a beetroot pulp extract with a higher total polyphenolic content of 255±10.48 mg GAE/100 g and antioxidant activity of 137±8.29 mg TE/100 g.

The L* parameter was recorded as 36.89±0.18, with higher values indicating a lighter color. The a* parameter measured 31.71±0.39, where positive values signify a shift toward red hues. The vibrant purple color of beetroot is primarily due to the presence of betalains. The positive b* value (4.82±0.10) indicates a shift toward yellow. Additionally, the color properties were analysed, revealing Chroma and Hue angle values of 31.25±0.25 and 0.15±0.03, respectively. Therefore, the beetroot powder was situated within quadrant I (+a*, +b*).

The observed discrepancies in results may be ascribed to changes in geographical origins or genotypes examined, extraction methods, and analytical procedures. Research has shown that BP powder is a valuable source of phytochemicals with antioxidant effects. These powders can be included in foods as a natural component to efficiently diminish the presence of agro-industrial residues.

Characterization of phytochemicals of red BPP-supplemented mayonnaise samples

The phytochemicals results are highlighted in Table 2. The incorporation of red BPP (1 and 2%) into the mayonnaise formulation resulted in a substantial elevation ($p<0.05$) in the concentrations of the bioactive molecules. Consequently, total betalains varied from 218.22±0.72 to 393.11±0.94 mg/100g, the flavonoid content ranged from 46.22±0.22 to 96.54 ±0.73 mg CE/100 g, while phenolic content ranged from 81.31±0.32 to 146.27±1.03 mg GAE/100 g in red BPP-supplemented mayonnaise samples.

Table 2. The phytochemical characteristics and antioxidant activity of the mayonnaise with red BPP (C- mayonnaise without the addition of red BPP, M1 and M2 - mayonnaise with the addition of 1% and 2% (*w/w*) red BPP)

Parameters	Mayonnaise samples		
	C	M1 (1%)	M2 (2%)
Total betalain content, mg/100 g dw	-	218.22±0.72b	393.11 ±0.94a
Total flavonoid content, mg CE/100 g dw	46.22±0.22c	78.31 ±0.62b	96.54 ±0.73a
Total polyphenol content, mg GAE/100 g dw	81.31±0.32c	102.09 ±1.02b	146.27 ±1.03a
Antioxidant activity, µMol TE/100 g dw	412.03 ±0.44c	766.13 ±0.96b	975.03 ±1.17a
DPPH Inhibition, %	14.80 ±0.08c	29.99 ±0.22b	38.76 ±0.23a

Different lowercase letters in a row highlight significant differences ($p<0.05$) between mayonnaise samples.

Furthermore, M1 and M2 samples exhibited significantly elevated quantities of bioactive chemicals ($p<0.05$) relative to the control sample. The mayonnaise samples enriched with red BPP exhibited more antioxidant activity than the control due to elevated quantities of bioactive components from the red BPP.

Raikos et al. (2016) demonstrated that mayonnaise samples fortified with processed (microwaved) beetroot extract exhibit elevated levels of bioactive components (betanin 337.9 mg/L; polyphenols 399.6 mg GAE/mL) and notable antioxidant activity (6.9 mol/L) in comparison to the control sample.



Figure 1. Comparative representation of the sensory attributes specific to mayonnaise samples: C- control mayonnaise sample; M1 and M2 - mayonnaise samples with 1 and 2% of red BPP

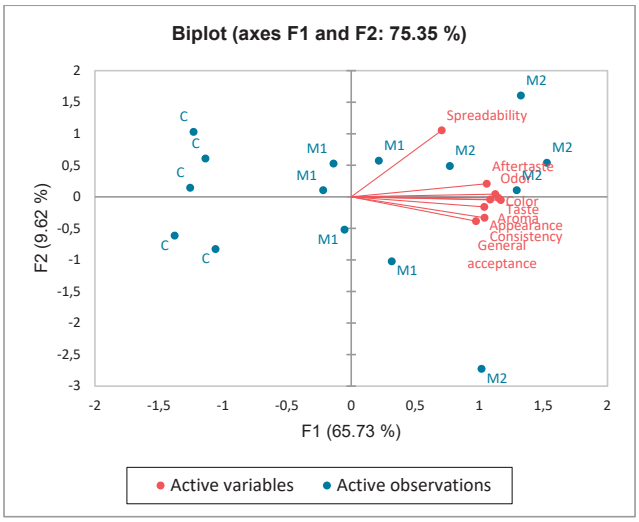


Figure 2. Biplot of Principal Component Analysis (PCA) depicting the positions of the three mayonnaise samples (C, M1, and M2) for the assessment of sensory qualities

Table 3. The physicochemical characteristics of the mayonnaise samples (C-control mayonnaise, M1 and M2 - mayonnaise with the addition of 1 and 2 % (w/w) red BPP)

Physicochemical characteristics	**Mayonnaise samples**		
	C	M1 (1%)	M2 (2%)
Moisture (g/100 g)	18.06 ± 0.06a	17.99± 0.11b	17.95± 0.21b
Lipids (g/100 g)	72.01 ± 0.21a	71.51 ± 0.19b	71.29 ± 0.13b
Proteins (g/100 g)	5.39± 0.16a	5.22± 0.03b	5.09± 0.02c
Carbohydrates (g/100 g)	2.61± 0.03c	3.17± 0.04b	3.28± 0.05a
Ash (g/100 g)	1.93± 0.03c	2.11± 0.06b	2.39± 0.08a
Energetic value %: kcal/100 g: kJ/100 g :	 680.09± 5.12a 2842.78± 5.12a	 677.15± 5.09b 2830.49± 5.09b	 675.09± 5.48b 2821.88± 5.48b

Different lowercase letters in a row highlight significant differences ($p<0.05$) between mayonnaise samples.

Table 4. Colorimetric parameters of the red BPP-supplemented mayonnaise samples

Parameters	**Mayonnaise samples**		
	C	M1 (1%)	M2(2%)
L*	78.60±0.55a	35.97±0.37b	32.15±0.35c
a*	2.27±0.20c	26.25±0.18b	30.68±0.30a
b*	38.07±0.39a	4.14±0.10c	4.52±0.06b
Chroma	38.14±0.38a	26.64±0.31c	30.96±0.29b
Hue angle	1.51±0.03a	0.17±0.02b	0.13±0.02b
ΔE	-	59.31±0.40b	64.16±0.39a

Different lowercase letters in a row highlight significant differences ($p<0.05$) between mayonnaise samples

Table 5. Textural parameters of the BPP-supplemented mayonnaise samples

Textural parameters	**Mayonnaise samples**		
	C	M1 (1%)	M2 (2%)
Firmness, N	0.66±0.02c	1.02±0.03b	1.67±0.05a
Adhesiveness, mJ	1.72±0.06b	5.13±0.17a	5.65±0.21a
Cohesiveness,-	0.80±0.02a	0.77±0.02a	0.75±0.01a
Chewiness, mJ	5.51±0.11c	9.86±0.16b	12.23±0.22a

Different lowercase letters in a row highlight significant differences ($p<0.05$) between mayonnaise samples

Table 2 indicates that red BPP enhances mayonnaise quality, as demonstrated by the substantial levels of betalains and polyphenols. Antioxidants enhance the mayonnaise's health benefits by scavenging free radicals.

Physicochemical characterization of red BPP-supplemented mayonnaise samples

The red BPP-supplemented mayonnaise samples were analyzed from a physicochemical point of view, and the results are presented in Table 3. The proximate analysis of mayonnaise with BP showed significant differences ($p<0.05$) between samples. Moisture content decreased with the addition of BP, while ash content increased as mayonnaise enrichment progressed. Additionally, the incorporation of beetroot powders led to an increase in carbohydrate content. The results were consistent with the findings of Lazar et al. (2022) in mayonnaise with beetroot peel.
Moreover, lipids and proteins decrease with the addition of red beetroot pomace. The energetic value differs from control with a decreasing trend.

Color evaluation of red BPP-supplemented mayonnaise samples

The incorporation of red BPP into the mayonnaise produced major color changes. The inclusion of BP significantly influenced the color of the mayonnaises, as indicated by the analysis of the color parameters.

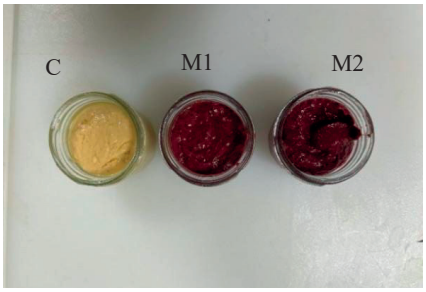


Figure 3. Images of the control mayonnaise sample (C); mayonnaise sample with 1% red BPP (M1); mayonnaise sample with 2% red BPP (M2)

The experimental mayonnaise sample exhibited the lowest lightness (L*) and yelowness (b*) values, alongside the highest redness (a*) values.

The value b* indicates a hue nearer to yellow. The red hue (a*) of beetroot is attributed to the presence of significant quantities of betalains. According to Table 4, a significant lightness decrease is observed with the BPP concentration increase and a tendency to red. The ΔE values demonstrated substantial variances across all mayonnaise samples. The overall color difference ΔE value in the mayonnaise samples increase rapidly with extract concentration, with ΔE reaching a higher value of 64.16±0.39 for mayonnaise made with 2% red BPP. A similar increase was observed by Lazar et al. (2022) in the mayonnaise samples enriched with beetroot peels.
Red beetroot pomace powder possesses significant coloring potency and can enhance the hue of the sauce, hence improving its consumer appeal.

Textural parameters of BPP-supplemented mayonnaise samples

Firmness (expressed in N) (Table 5) is defined as the maximum resistance force opposed by the sample during the first penetration cycle (Bourne, 2002). The firmness of mayonnaise is a crucial element as it affects both sensory attributes (mouthfeel) and usability (Chang et al., 2017). All samples demonstrated low firmness and cohesiveness values, alongside elevated adhesiveness and chewiness values, consistent with those documented in the literature (Di Mattia et al., 2015; Rojas et al., 2017). The results indicate that the incorporation of red BPP in mayonnaise samples resulted in greater firmness values compared to the control sample. This phenomenon results from the stabilizing molecules present in the incorporated plant material, such as pectin (Bai et al., 2017). Moreover, the incorporation of red BPP into the mayonnaise formulation enhanced adherence and chewiness, resulting in a refined and tender texture. Adhesion is defined as the energy required to remove the sample from the test device (Bourne, 2002). The lowest adhesion value, 1.72 mJ, was recorded for the control sample. The addition of red BPP in the composition of the mayonnaise samples contributed to improving the adhesion, giving the product a fine and creamy texture.
Cohesiveness is defined as the strength of the internal bonds between the constituent elements

of the mayonnaise (Bourne, 2002), which gives it its consistency. The higher the value, the more "sticky" or "cohesive" the mayonnaise. Thus the most cohesive mayonnaise was the control.
Instrumental texture analysis revealed that the addition of re BPP improves the textural characteristics of the mayonnaise in proportion to the concentration of added powder.

Sensory evaluation of red BPP-supplemented mayonnaise samples

The sensory assessment of the produced mayonnaise samples was performed on a nine-point hedonic scale. Figure 1 displays the average ratings obtained from the sensory assessment. Sensory analyses revealed that the mayonnaise formulated with the addition of 2% BPP (M2) received higher scores for most of the analyzed attributes (appearance, odor, consistency, taste, aroma, aftertaste, spreadability, and general acceptability) compared to the other mayonnaise formulations. Color is a key indicator of a product's quality that draws the attention of customers. The color of mayonnaise was significantly influenced by the concentration of red BPP ($p < 0.05$), with the M2 sample containing 2% red BPP receiving the highest color score. Enriching mayonnaise with BPP imparted an appealing red-purple hue, attributed to the increased presence of beetroot pigments (Figure 3). The amounts of red BPP incorporated into the mayonnaise influenced its odour, flavour, aftertaste, and spreadability.
Furthermore, it is noteworthy that the incorporation of higher concentrations of BPP into the mayonnaise led to a moderately elevated consistency rating.
Analyzing the results of the sensory evaluation of the value-added mayonnaise, it is noted that the mayonnaise variants with the addition of red beetroot powder were evaluated as having a balanced, pleasant taste, smell, and color corresponding to red beetroot, with a fine and creamy consistency. The findings are consistent with those of Raikos et al. (2016), who discovered that the incorporation of microwaved beetroot enhanced the color, texture, and overall acceptability of the mayonnaise.
In summary, the supplemented mayonnaise samples were well accepted by consumers. Figure 2 presents a PCA biplot that visually depicts the locations of the three mayonnaise samples (plain compared to those enhanced with BPP) according to the sensory qualities outlined in Figure 1. The initial principal component (PC1) represented 65.73% of the variance and comprised the two mayonnaise samples with BPP. The second component constituted 9.62% of the total and was comprised of control mayonnaise. Consequently, the two axes represented 75.35% of the total variation. The characteristics of aftertaste, odor, and spreadability exhibited a high correlation on the first axis, F1. Likewise, the characteristics of appearance, aroma, color, taste, consistency, and general acceptance were also identified as having a positive correlation within the same quadrant on the first axis, F1. Since all sensory attributes were exclusively linked to the same axis, F1, the control proved neutral. The biplot analysis enables the accurate positioning of the three mayonnaise samples according to their sensory attributes. It facilitates the distinction of sensory properties via positive correlations, as well as between sensory attributes and mayonnaise samples.

CONCLUSIONS

Beetroot pomace, a by-product of beetroot processing, has been effectively established as a significant source of bioactive chemicals, providing a unique approach to creating novel, value-added food products. The use of red BPP markedly enhanced the betalain, polyphenol concentrations and antioxidant efficacy in mayonnaise, hence augmenting its nutritional profile relative to the control sample.
The incorporation of red BPP enhanced the mayonnaise's aesthetic by augmenting redness and diminishing yellowness, attributable to the natural colors derived from red beetroot. Furthermore, it improved textural attributes, including firmness, adherence, and chewiness, yielding a softer and more appealing product consistency.
Sensory analysis verified that the incorporation of red BPP enhanced the color of mayonnaise without negatively impacting other sensory characteristics, such as aroma, aftertaste, spreadability, and overall approval among consumers. The use of beetroot by-products as a natural substitute for synthetic colors, flavorings, and antioxidants shows promise in

minimizing waste within the food industry. This corresponds with the tenets of a circular economy, promoting environmental sustainability and waste reduction. Red BPP functions as a natural and versatile component in the food sector, offering natural bioactive compounds and improving the visual and functional attributes of products. This method encourages the sustainable utilization of agro-industrial by-products and fosters creative food product fabrication.

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THE BOTTLED WATER QUALITY INFLUENCED BY PACKAGING MATERIALS AND STORAGE CONDITIONS - A MINI REVIEW

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Abstract

The quality of bottled water is significantly influenced by packaging materials and storage conditions, both of which have direct implications for human health. This mini-review explores how common packaging materials - such as polyethylene terephthalate (PET), polycarbonate (PC), glass, and aluminium affect water safety through chemical migration and leaching. Hazardous compounds like antimony, bisphenol A (BPA), phthalates, and microplastics can migrate from the container into the water, posing potential risks to endocrine, neurological, and reproductive health. Storage conditions further exacerbate contamination risks. High temperatures, prolonged storage periods, and exposure to light accelerate chemical leaching and microbial growth, increasing the likelihood of waterborne illnesses. This review highlights the critical need for regulatory frameworks that address both material selection and proper storage guidelines. Public education on optimal storage practices and further research into alternative packaging materials with minimal environmental and health impacts are essential to ensuring bottled water safety.

Key words*: chemical migration, contaminant exposure, environmental impact, food contact materials, water safety.*

INTRODUCTION

Water is vital for life since it aids in bodily metabolism and cell function. Potable water should have no taste, color, or odor and be free of any organisms or chemicals that could be harmful to human health. Drinking high-quality water is essential for human health and development. A consistent supply of safe drinking water is required to ensure healthy living among the residents of any geological location (Akharame et al., 2018). Human and animal activities have a significant impact on the availability and accessibility of fresh, clean water for human consumption.

Water is the only known liquid that efficiently hydrates the body and physically relieves thirst while simultaneously supplying a large amount of minerals (Sievers, 2005).

Water is the basic ingredient of living beings, and as the world's population grows by 1.1% every year, the need for water, including mineral water, is predicted to rise (Lee, 2011).

Drinking water is any type of water, in its natural state or after treatment, intended for drinking, food preparation, or other household purposes, regardless of its origin and whether it is supplied through a distribution network, from tanks, or distributed in bottles or other containers.

Bottled water has become a ubiquitous commodity worldwide, with its consumption steadily increasing due to urbanization, convenience, and perceived safety advantages over tap water (Zhang et al., 2021). Thus, in 2013, 52.8 billion liters of bottled water were consumed in the EU, which increased to approximately 63.2 billion liters in 2023 (Statista, 2024).

Numerous individuals prefer bottled water for several reasons, including convenience, aversion to the taste of tap water (attributable to chlorine and other impurities), apprehensions regarding tap water quality, its health benefits compared to carbonated soft drinks and other previously consumed beverages, and the diverse array of flavors and types of flavored water available. Growth in the culinary service sector, in conjunction with the expansion of the travel industry, has stimulated market growth. In addition, population growth, increasing disposable income, urbanization, and premiumization are additional factors (Zvěřinová et al., 2024).

The global bottled water market, valued at over $300 billion in 2023, reflects this trend, driven by consumer demand for portable and reliable hydration (Statista, 2024).

However, the quality of bottled water is not solely determined at the source; it is profoundly influenced by packaging materials and storage conditions, which can introduce chemical and microbial contaminants (Xu et al., 2021).

Plastic packaging, particularly polyethylene terephthalate (PET) and polycarbonate (PC), dominates the industry due to its lightweight and cost-effective properties. Yet, these materials are associated with the migration of hazardous compounds such as antimony, bisphenol A (BPA), and phthalates into water, especially under suboptimal storage conditions (Franz & Welle, 2021). For instance, antimony, a catalyst used in PET production, can leach into water at levels exceeding safety thresholds when bottles are exposed to high temperatures (Reimann et al., 2012). Similarly, BPA, an endocrine disruptor found in PC containers, has raised health concerns despite regulatory limits (Chen et al., 2023).

Beyond chemical risks, microbial contamination poses another challenge. Improper handling, prolonged storage, or compromised seals can determine bacterial growth, like *Pseudomonas* spp. and *E. coli*, which thrive in nutrient-poor environments (Diduch et al., 2016). Studies have shown that even "minimally processed" bottled water can harbour microbial communities after extended shelf life (Dhaka et al., 2022).

This mini-review critically evaluates recent research (2020-2024) on how packaging materials (PET, PC, glass, and alternatives) and storage factors (temperature, light, duration) impact bottled water quality.

MATERIALS AND METHODS

A targeted literature study was performed on PubMed, Scopus, and Google Scholar to locate original peer-reviewed articles regarding the purity of bottled water, food contact materials, and water safety. The searched key words included "bottled water", "drinking water", "tap water", "food packaging", "storage conditions", "safety", and "quality" in various permutations. Furthermore, the reference lists of the discovered papers were scrutinized to uncover any further publications. Moreover, procedural blank samples were necessary. Furthermore, we sought statistical data regarding bottled water use on Eurostat, Statista, and other available platforms. Experimental research and non-English publications were omitted.

RESULTS AND DISCUSSIONS

Bottled water consumption and consumer preferences

Accordingly, to Order 7/2023, drinking water must be healthy and clean, with no microorganisms, substances or parasites that, by their number or concentration, may be hazardous to human health.

A fundamental component of food safety and security is the access to safe and healthy drinking water which also enhances social and economic development of the societies (WHO, 2021).

The criteria for selecting a water source for potable use are: quantitative (the source must provide the required amount of water throughout the year); qualitative (the source must be in line with the specific regulatory standards for water intended for drinking), technical (referring to the method of source extraction, treatment recommendations, and the equipment used) and economic (includes the costs of design, construction, and operation (Bodor et al., 2021).

Ensuring high-quality drinking water involves management strategies that guarantee the control of hazardous components in the water.

The consumption of water from the distribution network is limited or very limited, being replaced by bottled water consumption as a general trend. The main reason for rejecting tap water is the significantly altered organoleptic properties due to excessive iron and manganese content. People choose tap water because of its low cost, but at the same time, they fear its quality. Even if it is microbiologically clean and meets physicochemical standards, the main issue in certain areas is the piping system. The old and potentially contaminated pipes through which drinking water reaches consumers are a concern, leading many people to opt for bottled water instead (Zvěřinová, 2024).

Romania has over 2,000 mineral springs, whose chemical diversity reflects the highly complex geological conditions that have influenced their formation. Most springs contain carbonated mineral waters, originating from post-volcanic phenomena associated with Neogene magmatism.

The most significant mineral water deposits in Romania, which are used for bottling, are located in mountainous areas and intramountain depressions, far from pollution sources typically found in industrial zones or regions with intensive agriculture (Feru, 2012).

According to the National Society of Mineral Waters study, Romania possesses 60 percent of Europe's mineral water deposits, although barely one-fifth of these resources are utilized (FRD Center Market, 2016). According to Directive 2009/54/EC, Romania recognizes 71 varieties of natural mineral waters (European Commission, 2020). The origin and location of the analyzed mineral waters indicate that the majority of brands are located in Harghita, Suceava, and Covasna counties (Figure 1) (Bodor et al., 2021).

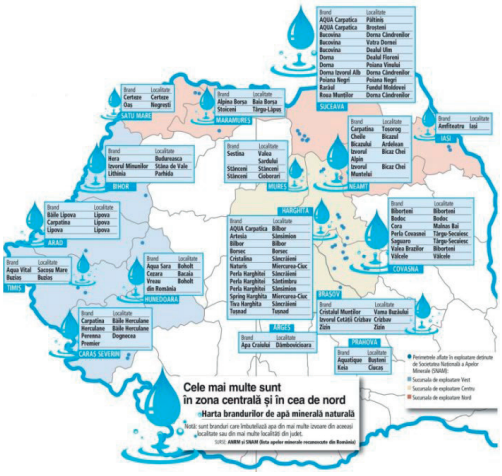


Figure 1. Geographical representation of mineral water sources in Romania (https://bihornews.ro/wp-content/uploads/2022/08/y-15-768x730.jpg)

Natural mineral water is microbiologically pure water that originates from a groundwater table or an underground aquifer and is sourced through one or more natural springs or boreholes (HG 1020/2005).

The attributes that could provide advantageous health benefits of natural mineral water require assessment: (a) from the geological, hydrological, physicochemical, microbiological characteristics; clinical and physiological perspectives; (b) according to criteria related to research methodology; (c) in accordance with scientifically validated techniques sanctioned by the appropriate government.

According to the definition, natural mineral water should be of subterranean origin. Due to its natural protection, reinforced by human intervention through the establishment of strictly regulated activity zones around the springs, natural mineral water has to be microbiologically pure, with no content of contaminants, and maintain a stable physicochemical composition over time. Unlike regular drinking water, natural mineral water has no set limits for its major chemical constituents (Feru, 2012).

The primary criteria for the predominant categorization include *geological origin* (the source of water), *chemical composition* (the presence and stability of minerals), and *carbonation* (the inclusion of carbon dioxide), determining whether the water is sparkling or still. Moreover, all bottled waters can be classified into two primary categories: natural and treated, although this simplistic classification may not adequately represent the diverse array of bottled water varieties. This analysis focused mostly on bottled water varieties that exhibit minimal or no flavor differentiation from municipal tap water. These varieties are classified into the following categories.

Natural mineral water (Figure 2), derived from subsurface sources that are naturally protected from contamination, is characterized by the presence of minerals and trace elements. The elemental composition differs by geographical region, lending a unique flavor and commercial label to the water (e.g., Evian, Fiji Natural Artesian).

No definitive statement or definition exists for the classification of water as a "mineral." Despite the fact that the European Bottled Water Standard is 50 ppm, the International Bottled Water Association (IBWA) standard is 250 ppm. Bottled water's mineral content must

stay unchanged from source to consumer, without any treatment.
The mineral amount and content, provenance and source must be explicitly specified.
Treated water. Purified, processed, prepared, or table water are all terms that are occasionally used to describe treated water. The information as to its origin is irrelevant to its branding, and there are no legal obligations to disclose it. An unconfined aquifer, a surface water source, or a municipal or community water system could be the source. To ensure its safety for consumption, this water undergoes necessary remedies, including disinfection. The treatment may involve the use of deionization, distillation, reverse osmosis, or other methods.
Regarding the bottled (packaged) water consumption in European Union, in 2022, it can be observed (Figure 4) that Italy has the highest amount (249L/capita/year), doubled compared to the EU average consumption, followed by Germany, Hungary, Greece, France, Portugal and Spain. In Romania, the annual consumption was 123L/capita).
Still bottled water is preferred by 64% of consumers, with thirty eight percent of consumers indicating their preference for sparkling waters (Figure 2).

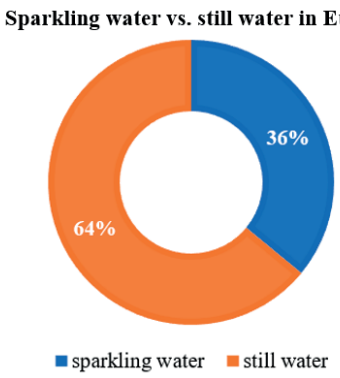


Figure 2. Sparkling water versus still water consumption in the EU in 2022
(https://naturalmineralwaterseurope.org/statistics/)

48% of EU consumers, consider bottled water as their most liked and appropriate way of healthily hydrating. In the meantime, 39% of European consumer choose to consume soft drinks. Bottled water maintains a consistent market share in comparison to other packaged non-alcoholic beverages, according to the most recent statistics (Figure 3).

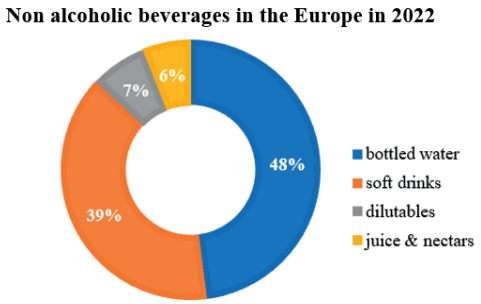


Figure 3. Non-alcoholic beverages in the EU, 2022
(https://naturalmineralwaterseurope.org/statistics/)

In a ten-year EU water consumption analysis (Figure 5), it can be seen an increasing of the water quantity per capita, with almost same amount in 2022 and 2023 (Statista, 2024).
Consumers also appear to clearly prefer natural mineral water in their choice of drinking water, with spring waters coming in second place.
The main reasons for people choosing bottled water are: preference for bottled water taste, convenience, dislike the quality and sensorial perceiving of tap water (due to chlorine and other content), concerns about the quality of tap water, consideration that is healthier than carbonated soft drinks and other beverages that consumers were drinking before, the availability of different kinds of flavoured water. Growth in the travel industry, together with the rising food service sector, has also had an impact on market growth. Other causes include population growth, rising disposable income, rapid urbanization, and premiumization.

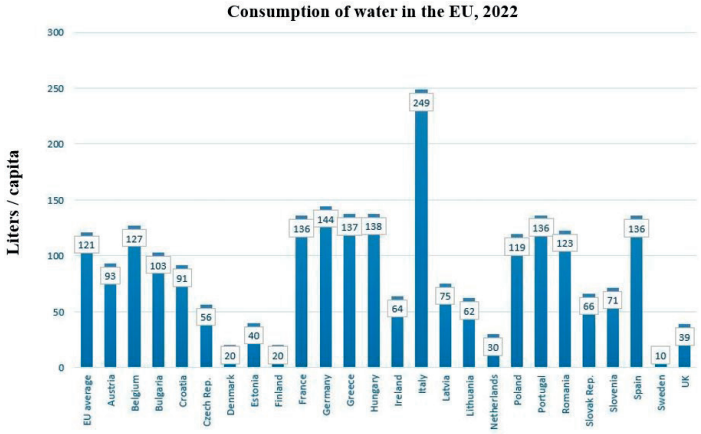


Figure 4. Water consumption in the EU in 2022, Liters per capita / year (https://naturalmineralwaterseurope.org/statistics/)

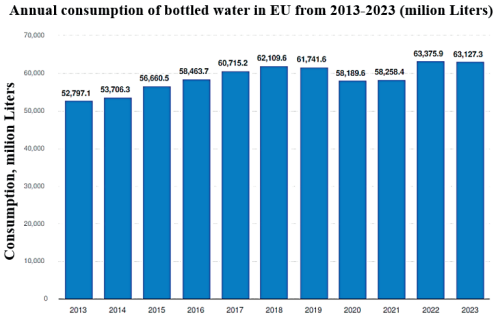


Figure 5. Annual consumption of bottled water in EU from 2013-2023 (Statista, 2024)

Bottled water quality

The description of natural mineral waters is "natural mineral water" or, in the case of an effervescent natural mineral water as defined in "naturally carbonated natural mineral water", "natural mineral water fortified with gas from the spring" or "carbonated natural mineral water" as of the EU Directive 2009/54.

The labels must include the analytical composition, the spring's place of extraction (including its name), and the treatments applied.

There are no upper or lower limits for minerals contents in natural mineral water; instead of the tap drinking water, that are strictly regulated as regards the fixed residue at 180°C. Mineral waters are also classified by other physical parameters, like pH, temperature at source and hardness. In terms of pH, mineral waters are classified as acid water (pH = 7). Waters can be classified as frigid (<20°C), hypothermal (20-30°C), mesothermal (30-40°C), or hyperthermal (>40°C) based on the water temperature at the source. According to Directive 2009/54/EC, mineral waters may be classified as very soft (0-100 mg/L of $CaCO_3$), soft (100-200 mg/L of $CaCO_3$), hard (200-300 mg/L of $CaCO_3$), or very hard (> 300 mg/L of $CaCO_3$), which is indicative of the presence of alkaline earth metals.

As stated in the abovementioned Directive, mineral waters can be labelled with different claims (criteria) shown in Table 1.

The transportation of mineral water has evolved significantly over time, from oak barrels carried by carts and bottling in clay vessels to a large-scale industry today. In the past, daily production was limited to around 2,000 liters per day, whereas now, the industry operates on a much larger scale.

Table 1. Indications and criteria for mineral water (EU Directive 2009/54/ Annex III)

Indications	Criteria
"Low mineral content"	Mineral salt content, expressed as a fixed residue, < 500 mg/L
"Very low mineral content"	Mineral salt content, expressed as a fixed residue, < 50 mg/L
"Rich in mineral salts"	Mineral salt, expressed as a fixed residue, >1500 mg/L
"Contains bicarbonate"	Bicarbonate > 600 mg/L
"Contains sulphate"	Sulphate > 200 mg/L
"Contains chloride"	Chloride > 200 mg/L
"Contains calcium"	Calcium > 150 mg/L
"Contains magnesium"	Magnesium > 50 mg/L
'Contains fluoride"	Fluoride > 1 mg/L
"Contains iron"	Bivalent iron >1 mg/L
"Acidic"	Free carbon dioxide > 250 mg/L
"Contains sodium"	Sodium > 200 mg/L
"Suitable for a low-sodium diet"	Sodium < 20 mg/L

The first bottling in glass containers in Europe took place in 1806 in Romania, marking the beginning of a continuous transformation process. Over recent years, the bottling of mineral water has been subject to numerous analyses and studies, while the market has diversified with an increasing number of competitors (Feru, 2012).

Water bottling is carried out in containers made of glass, polyethylene terephthalate (PET), and high-density polyethylene (HDPE). Even though HDPE packaging is chemically more stable than PET packaging, the migration of chemical compounds still occurs.

Currently, countries with varying levels of economic advancement worldwide consume a wide range of types and brands of bottled/packaged drinking water. This water is contained in hermetically sealed containers of varying compositions, forms, and capacities (e.g., bottles, water dispensers, sachets) and is safe for direct consumption (FAO/WHO, 2007).

From its humble beginnings as a specialty product, bottled water has grown into a global phenomenon. It has a long history in Europe, dating back to the 16th century when glass bottles of natural mineral water were sold as a special occasion luxury drink. With the invention of industrially carbonated water in 1806 and the introduction of the first bottled mineral water for public use in the United States in the second half of the 18th century, the use of packed / bottled water stayed small until the second part of the 20th century, despite the decreasing of the cost of glass. This started to alter in the late 1960s and early 1970s, when the United States started to import more mineral water from Europe and a new focus in overall fitness began to take hold. Bottled water sales were already booming when polyethylene terephthalate (PET) was launched in the late 1970s United Nations University Institute for Water, Environment and Health (Bouhlel et al., 2023).

The role of packaging materials on bottled water quality - components migration

The main plastics used for water bottles are Polyethylene Terephthalate (PET), and High-Density Polyethylene (HDPE).

Polyethylene Terephthalate (PET) is used mostly for bottled water containers and the primary migrants consist in: *antimony* (Sb) used as a catalyst in PET production. Its migration increases with temperature (Franz & Welle, 2021). At 25°C: ~0.5 ppb (below WHO limit of 20 ppb) but at 60°C: up to 10 ppb (Reimann et al., 2012). Another migrant component is *acetaldehyde*, a degradation product affecting taste (Chen et al., 2023) and *phthalates* (DEHP, DBP), which are present in recycled PET due to contamination (Muncke et al., 2020).

The migration is influenced by UV exposure (which accelerates PET degradation, increasing Sb leaching (Geueke et al., 2022), and by the reuse of bottles (producing scratching and heating enhance chemical release (Licciardello, 2024).

High-Density Polyethylene (HDPE) used for milk flask and detergent containers. "Non-intentionally added substances" (NIAS) like antioxidants and oligomers have been detected.

The primary migrants from HDPE are non-intentionally added substances (NIAS) like antioxidants (e.g., Irganox 1076) degrade into unknown byproducts (Biedermann et al., 2021) and oligomers (small polymer chains) detected in stored water (Chen et al., 2023).

The volatile organic compounds (VOCs) are another migrants, residual solvents from manufacturing (Schaider et al., 2017). The migration is influenced by the fatty foods/liquids: Increase leaching of non-polar compounds (Muncke et al., 2020).

Nowadays, there are emerging contaminants of concern like *microplastics and nanoplastics* (PET and HDPE bottles shed microplastics (~50–300 particles/L, WHO, 2021), *per- and polyfluoroalkyl substances (PFAS)* found in grease-proof coatings of paper-based packaging (Biedermann et al., 2021), linked to cancer and immune suppression (Muncke et al., 2020) and *bisphenol A (BPA) and alternatives (BPS, BPF)*, leached from epoxy-lined aluminium cans (Geueke et al., 2022), that may have similar endocrine effects (Licciardello, 2024).

Elevated temperatures accelerate the leaching of antimony (Sb), phthalates, acetaldehyde, and formaldehyde from PET bottles into water (Franz & Welle, 2021; Dąrowska et al., 2003). Antimony trioxide (Sb_2O_3), a PET production catalyst, migrates into water, especially at temperatures >50°C, nearing regulatory limits (Shotyk et al., 2006). UV exposure degrades PET, increasing volatile organic compounds (VOCs) like formaldehyde (Yutaka et al., 2021). Higher storage temperatures (e.g., 40-60°C) significantly increase contaminant migration (Morin-Crini et al., 2022).

Similarly, Re Depaolini et al. (2020) reported higher levels of acetaldehyde, a degradation byproduct of PET in water kept at a temperature of 40°C compared to 25°C. Ahmed et al. (2021) tested multiple heating sources (sun, oven, microwave) and freezing, on two brand PET bottles and their influence on water quality and components migration providing comprehensive comparative data. Related to temperature-driven chemical migration, they observed that the pH decreased (especially at temperature higher than 50°C and by frosting). Total Dissolved Solids (TDS) rose by up to 18% (B1) and 6.5% (B2) with heating, though remaining within permissible limits. Fluoride & Chloride: Microwave heating at 50-70°C increased fluoride beyond WHO limits (up to 2.47 mg/L vs. 1.5 mg/L). Chloride levels fluctuated but decreased sharply in B2 with microwave heating. Heavy Metals: Minimal antimony (Sb) leaching (contrary to prior studies), but copper (Cu) and zinc (Zn) were detected, especially with oven heating at 70°C.

Endocrine-Disrupting Compounds (EDCs) Bisphenol A (BPA), alkylphenols, and phthalates migrate from PET, especially under heat (Amiridou & Dimitra, 2011; Wagner & Oehlmann, 2009) and estrogenic activity was detected in 60-78% of bottled water samples, linked to both packaging and groundwater sources (Pinto & Reali, 2009).

The influence of storage conditions on bottled water quality

Byproducts such oligomers and diethylene glycol are created during the thermal breakdown of PET. According to Bach et al. (2012b), PET samples that were heated to high temperatures contained volatile organic compounds like carbon monoxide, aldehydes (formaldehyde, acetaldehyde, benzaldehyde), aromatic hydrocarbons (benzene, toluene, ethylbenzene, and styrene), esters (vinyl benzene, methyl acetate), methanol, and acetophenone. Studies have also investigated the migration of antimony from PET bottles into bottled water, analyzing factors such as temperature, storage time, water pH, and bottle color. It was found that high temperatures and long storage periods promote antimony release, while carbonated water accelerates this process due to its lower pH. Research conducted in Europe and Canada confirmed the presence of antimony in PET-bottled water, with average concentrations ranging from 0.343 to 2 µg/L, whereas water bottled in glass containers showed no contamination. Additionally, transparent PET bottles appear to release more antimony than colored ones, though some studies reported opposite results. Research has also shown that antimony migration depends on the contact surface area, being higher in smaller bottles. Moreover, water contamination originates not only from PET but also from the bottling process. These findings highlight the need for strict regulations regarding the use of

PET in drinking water packaging (Bach et al., 2012b).

Water bottled in PET has been found to have estrogenic and genotoxic effects. These toxicological consequences may be caused by chemical combinations in bottled water, according to Bach et al. (2012a). The migration of formaldehyde, acetaldehyde, and antimony from PET into water is clearly linked (Bach et al., 2012a).

Polyethylene terephthalate (PET) is the most often used plastic in the production of packed drinking water containers, and it takes 400 years for it to breakdown spontaneously in nature. It is used for bottled water packaging due to its lightweight, durability, and recyclability. However, storage conditions, particularly temperature, light exposure, and duration, can significantly impact water quality and safety by promoting chemical migration, microbial growth, and PET degradation.

Impact of Light Exposure

Sunlight (UV radiation) degrades PET, increasing the release of volatile organic compounds (VOCs) and altering water taste. Akharame et al. (2018) observed that PET bottles exposed to sunlight for 30 days showed higher concentrations of formaldehyde and acetaldehyde compared to dark-stored samples.

Storage conditions were investigated related to their influence on bottled water quality. It was noted that higher storage temperatures (e.g., 40-60°C) significantly increase contaminant migration (Schmid et al., 2008). Sunlight exposure promotes PET degradation and VOC release (Nawrocki et al., 2002) and long-term storage (>6 months) raises antimony and acetaldehyde levels (Montserrat, 2020).

pH decreased significantly with prolonged sunlight exposure (e.g., from 6.5 to 4.7 after 28 days), attributed to increased acidity from PET degradation. TDS and EC increased with sunlight exposure (TDS: 5-122 mg/L to 5.5-139 mg/L; EC: 10-242 µS/cm to 17-291 µS/cm), likely due to leaching of ions from PET bottles (Akharame et al., 2018).

Microplastic is a collection of various distinct chemical components. It is composed of various molecules with varying molecular structures, shapes, sizes, and polymers (Rochman et al. 2019). Microplastic is one of the most complicated pollutants created by mankind (Arias-Andres & Rojas-Jimenez, 2022).

Regulatory

Directive 2009/54/EC of the EP describes the rules for exploitation and marketing of natural mineral waters (Table 2).

The primary objectives of any regulations concerning mineral waters are to protect consumer health, prevent misleading information, and ensure fair marketing. It must be guaranteed that natural spring waters retain, at the time of commercialization, the characteristics that led to their recognition as such. Therefore, the packaging containers used must have appropriate sealing systems.

Under the prevision of this Directive, *carbonated natural mineral water* is described as “water to which has been added carbon dioxide (CO_2) of an origin other than the water table or deposit from which the water comes and naturally *carbonated natural mineral water* as water the CO_2 content of which from the spring after decanting, if any, and bottling is the same as at source”. This takes into account, where appropriate, the restoration of a quantity of CO_2 from the same water or deposit equivalent to that was lost during processing. The declaration of the composition of natural spring water should be mandatory to guarantee consumer information.

The breakup of unstable elements, like sulphur or iron and their compounds, through filtration or decantation, potentially preceded by oxygenation, is the only treatment that natural mineral water can endure in its natural state at the source.

Treatment of specific natural mineral waters with ozone-enriched air to eliminate iron, manganese, sulphur, and arsenic compounds, provided that the treatment does not modify the water's composition in terms of its characteristic constituents that confer its properties. The removal of unbound carbon dioxide through exclusively physical processes, whether complete or partial.

Table 2. Legislation for mineral, spring and bottled water

Product	Legislation	Other Regulations on Food Safety and Food Hygiene
Natural Mineral Water	"Directive 2009/54/EC on the exploitation and marketing of natural mineral waters"	"Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety" "Regulation (EC) No 852/2004 on the hygiene of foodstuffs" "Regulation (EC) No882/2004 on official controls performed to ensure verification of compliance with feed and food law, animal health and animal welfare" "Commission Directive 19/2004/EC on plastic materials and articles intended to come into contact with foodstuffs" "Commission Regulation (EC) No 282/2008 on recycled plastic materials and articles intended to come into contact with foodstuffs" "Regulation 178/2002/EC" "Regulation 852/2004/EC" "Regulation 882/2004/EC" Guidelines for the development of EU "Guides to Good Practice for hygiene or for the application of the HACCP principles"
Spring Water	"Regulated partly by Directive 2009/54/EC on the exploitation and marketing of natural mineral waters "Directive 98/83/EC on the quality of water intended for human consumption"	
Bottled Drinking Water	"Directive 98/83/EC relating to the quality of water intended for human consumption". **"Codex Standard for Natural Mineral Waters** (108–1981 adopted 1981, amendment 2001, revisions 1997, 2008)" "Recommended International Code of Hygienic Practice for the Collecting, Processing and Marketing of Natural Mineral Waters (CAC/RCP 33-1985, revised 2011)" "General Standard for Bottled/Packaged Drinking Waters (other than Natural Mineral Waters) (227-2001)" "Code of Hygienic Practice for Bottled/Packaged Drinking Waters(other than Natural Mineral Waters) (CAC/RCP 48-2001)" **"WHO Guidelines for Drinking Water** Quality is the reference basis upon which all bottled waters legislation derives from. These international norms on water quality and human health in the form of guidelines are used as the basis for regulation and standard setting, in developing and developed countries world-wide."	

To be recognized as natural mineral water, a source must meet strict quality and safety criteria. The Romanian recognized sources of mineral waters are presented in Table 3.

Table 3. List of natural mineral waters recognized by Romania (Eurlex, 2025)

Trade Description	Name of source	Place of exploitation
Alpina Borşa	Izvorul nr.1, bis Izvorul nr.2	Baia Borşa (Maramureş County)
Amfitreatru	Izvorul 3 Copou	Iaşi (Iaşi County)
Apa Craiului	Izvorul nr.5 Gălgoaie	Dâmbovicioara (Argeş County)
Aquatique	Izvorul Bușteni	Bușteni (Prahova County)
Aqua Carpatica	Izvorul Bejenaru	Păltiniş (Suceava County)
	Izvorul Haja	
	F2 Păltiniş	
	Izvorul Ichim nr.1 şi 4	Gălăuţaş, Bilbor (Harghita County)
	Aqua 2	
	Aqua 3	
	Aqua 4	
	Aqua	Broşteni (Suceava County)
Aqua Sara	F 4750	Bodolt (Hunedoara County)
Aqua Vital	Sacoşu Mare	Sacoşu Mare (Timiş County)
Apă Minerală Natural Carbogazoasă Stoiceni	Sursa F1	Stoiceni Târgu Lăpuş (Maramureş County)
Armonia Starea Ta De Bine	F2 Măieruş	Măieruş (Braşov County)
Artesia	FH Artezia	Sânsimion (Harghita County)
Băile Lipova	F11	Lipova (Arad County)
Biborţeni-Bătani	F1 SNAM	Biborţeni-Bătani (Covasna County)
Biborţeni	F7	Biborţeni (Covasna County)
Bilbor	F1 SNAM	Bilbor (Harghita County)

Bodoc	Izvorul Mathid F13 RAMIN	Bodoc (Covasna County)
Borsec	Borsec	Borsec (Harghita County)
	Făget Borsec	
	F1C	
Bucovina	C7	Dorna Candrenilor (Suceava County)
	F4 SNAM ROŞU	
	F2 RAMIN ROŞU	
	F1, F2, F3, F3 bis, F1 bis, F1 bis SNAM, F2 SNAM	
Buziaş	F II bis	Buziaş (Timiş County)
Carpatina	C1	Domogled-Băile-Herculane (Caraş-Severin County)
	F1ISLGS	Toşorog (Neamţ County)
Certeze	Certeze	Certeze (Satu-Mare County)
Cezara	F3, F4	Băcaia (Hunedoara County)
Cheile Bicazului	Bicazul Ardlean, forajul FH 1	Bicazul-Ardelean (Neamţ County)
Cora	F1 SNAM	Mălnaş Băi (Covasna County)
Cristalina	FH Artezia 1	Sânsimion (Harghita County)
Cristalul Munţilor	Izvorul Pârâul Rece	Vama Buzăului (Braşov County)
Dorna	FH2, FH3 Floreni	Dealul Floreni (Suceava County)
	F5	Poiana Vinului (Suceava County)
	F2bPN, F3PN, F1cPN	Negrişoara-Poiana Negri (Suceava County)
	Fd	Poiana Negri (Suceava County)
Dorna Izvorul Alb	Captarea1, 2, 2 bis, 2a, 2b, 3, Izvorul 5, 6, 7, 10, Foraj 1IA,F1bis VB	Dorna Candrenilor(Suceava County)
Hera	Izvor Hera(Cuciului)	Budureasa (Bihor County)
Herculane	C2	Domogled-Băile-Herculane (Caraş-Severin County)
Izvorul Alpin	Izvorul Alpin	Bicaz Chei (Neamţ County)
Izvorul Cetatii Crizbav	Izvor	Crizbav (Braşov County)
Izvorul Minunilor	Izvorul Minunilor	Stâna de Vale (Bihor County)
Izvorul Muntelui	Izvorul Muntelui	Comuna Bicaz Chei (Neamţ County)
Keia	Izvorul Zăganului	Ciucaş (Prahova County)
La Fantana	Şeştina	Valea Şardului (Mureş County)
Lipova	F8E, F9 bis,	Lipova (Arad County)
Lithina	Forajul FH2	Parhida (Bihor County)
Naturis	FH1	Miercurea-Ciuc (Harghita County)
Oaş	Certeze Negreşti I	Negreşti(Satu-Mare County)
Porenna Premier	Calina1	Dognecea (Caraş-Severin County)
Perla Apusenilor	FH2 Chimindia	Chimindia-Deva(Hunedoara County)
Perla Covasnei	F1 SNAM	Târgul Secuiesc (Covasna County)
Perla Harghitei	F1 SNAM, F2 SNAM, F1 ISPIF	Sâncrăieni (Harghita County)
Perlaharghitei	FH2 Sântimbru	Sântimbru (Harghita County)
Perlaharghitei	FH Artezia 2	Sânsimion (Harghita County)
Rarâul	Puţul Sebeş FH1	Fundul Moldovei (Suceava County)
Spring Harghita	FH2M	Miercurea-Ciuc (Harghita County)
Stânceni	Stânceni	Stânceni(Mureş County)
Stânceni	Ciobotani	Ciobotani(Mureş County)
Tărâmulapelor	F3 ISPIF	Târgul Secuiesc (Covasna County)
Tiva Harghitei	F8 ISPIF	Sâncrăieni (Harghita County)
Tuşnad	Tuşnad, FH 35 bis	Tuşnad(Harghita County)
Vâlcele	Elisabeta	Vâlcele (Covasna County)
Vreau Din România	F6 ISPIF	Boholt (Hunedoara County)
Zizin	Sursele Zizin	Tărlungeni (Braşov County)

The water must originate exclusively from an underground source, protected from any contamination, and maintain a stable chemical composition. It must not be influenced by human activities and must contain minerals and trace elements with beneficial health effects. Additionally, the mineral composition and physicochemical properties must remain constant over time, and the water cannot be subjected to chemical treatments for purification, it must be naturally pure at the source.

To guarantee these properties, the water must undergo a rigorous process of physicochemical and microbiological analysis over an extended period, in compliance with national and European safety and quality standards.

The bottling of mineral water in Romania is a regulated process carried out in accordance with national and European safety and quality standards, considering that natural mineral water must retain the characteristics that define it at the source, without being subjected to chemical treatments.

CONCLUSIONS

Mineral water composition varies geologically: Romania's mineral waters (e.g., from Harghita, Covasna) have unique mineral profiles, but their stability depends on packaging integrity during storage.

Consumer preferences drive market trends: 48% of EU consumers prefer bottled water for convenience and perceived safety, despite tap water often meeting equal quality standards.

Packaging material significantly impacts water quality: PET and HDPE bottles leach chemicals (e.g., antimony, BPA, phthalates) into water, especially under heat or UV exposure. Glass and aluminium are safer but less commonly used due to cost and weight.

temperature and light accelerate contaminant migration: storage at >40°C or in sunlight increases leaching of antimony, acetaldehyde, and microplastics from PET bottles, altering taste and safety (e.g., formaldehyde levels rise by 30% after 28 days of UV exposure).

Microbial risks are linked to storage conditions: prolonged storage or poor sealing promotes bacterial growth (e.g., *Pseudomonas* spp.), though sunlight can reduce coliforms via natural disinfection.

Regulatory gaps exist for emerging contaminants: current EU directives (e.g., 2009/54/EC) lack limits for microplastics, PFAS, and endocrine disruptors like BPA alternatives (BPS, BPF).

PET degradation releases harmful byproducts: high temperatures generate VOCs (e.g., benzene, formaldehyde) and oligomers, with recycled PET posing higher contamination risks due to prior use.

pH and TDS are key indicators of quality changes: sunlight exposure reduces pH (increasing acidity) and raises TDS due to ion leaching, though values often remain within WHO limits.

Antimony leaching is a persistent issue: Sb levels in PET-bottled water can approach regulatory limits (5 µg/L in EU) at 60°C, with smaller bottles showing higher migration rates.

Public awareness and alternative materials are critical: education on proper storage (e.g., avoiding heat/sunlight) and investment in biodegradable or inert packaging (e.g., plant-based plastics) are needed to mitigate health risks.

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INVESTIGATING THE BENEFITS OF PLUM POMACE POWDER AS A NUTRITIOUS ADDITION TO YOGURT

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Abstract

The objective of the study was to examine the impact of enriching yogurt with plum pulp powder (PP) on the physicochemical, antioxidant, and sensory traits of the final product. The milk was sourced locally, and the bioactive ingredients were extracted via the ultrasound-assisted method. The findings indicated a significant enhancement in the antioxidant capacity of the fortified yogurts, achieving values of up to 20.98±1.19 µmol TE/g d.w. at the highest concentration of PP (BPP3). The incorporation of PP enhanced the texture, colour, and smell of the yogurts, which was favourably received by the panellists. The incorporation of PP also resulted in a uniform pink hue and enhanced firmness of the yogurt, without adversely affecting acidity or syneresis during storage. While the most significant advantageous effects were identified at a concentration of 12% PP, an increase in acidity and whey separation was also recorded with time. The results obtained underscore the potential of plum powder as a bioactive component for the formulation of functional dairy products, hence facilitating the advancement of functional products and supporting the principles of a circular economy.

Key words*: antioxidant activity, dairy products, functional yogurt, pigments, plum pomace.*

INTRODUCTION

A dairy product with a straightforward yet distinctive composition, yogurt is full of different components that provide health benefits. It is an oil-in-water emulsion, with proteins, carbohydrates, and minerals in the water phase and fatty droplets in the oil phase (Kamal et al., 2018). The presence of lactic acid bacteria cultures, such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, distinguishes yogurt from other dairy products. Lactic acid, which is produced by these bacteria, lowers the pH and inhibits the formation of potentially harmful pathogens in the yogurt (Nateghi et al., 2018; Santos et al., 2024).

The food ingredients market has expanded significantly owing to the increasing customer demand for a broader range of food products. A multitude of these elements is provided in powdered form, and the technologies used for their production are gaining significance as their functionality and quality progressively align with the efficiency of manufacturing processes (Guiné et al., 2020). Powdered fruit ingredients can enhance the color and flavor of various foods and pharmaceutical products while providing additional health-related benefits (Orădan et al., 2024). Fruits and other natural plant components are exceptional sources of bioactive compounds (Vlaicu et al., 2023). Plums have garnered significant attention among fruits due to their economic relevance in fruit production and their cultivation on a substantial portion of the agricultural area within the European Union (Rusu et al., 2024).

Recent studies have examined the impact of incorporating plum pulp into yogurt. A study by

Arora et al. (2021) evaluated the sensory qualities and physicochemical properties of yogurt enhanced with varying percentages of plum pulp (*Prunus domestica*) at 10%, 15%, and 20%, revealing several changes in the final product.

Consequently, the examined samples exhibited an increase in acidity and a reduction in pH value after storage, indicating heightened metabolic activity of lactic acid bacteria. The yogurt with 10% plum pulp received the top ratings for flavor, aroma, color, and consistency, indicating strong consumer approval. In this context, plum pulp may serve as a valuable functional component for incorporation into yogurts, enhancing both nutritional value and sensory appeal, and can be utilized to create natural, functional, and health-promoting dairy products.

Yogurt, an extremely common fermented dairy product, exists in numerous varieties and is recognized for its nutritional and nutraceutical advantages. The incorporation of natural or modified compounds into yogurt has gained popularity as an option to augment its functioning and nutraceutical properties. Reports indicate the incorporation of many natural ingredients in yogurt production, such as date extract, grape skin, mulberry fruit and leaf powder, lentil flour, assorted fibers, lemon and mint essential oils, and various fruit press powders (Bankole et al., 2023; Stoica et al., 2024).

Extracts from fruits and other plant components significantly enhance the nutritional and functional value of yogurts by augmenting antioxidant activity, bioactive component content, and health benefits, as indicated by a studies conducted by Wajs et al. (2023) and Gavril et al. (2024). Nonetheless, variations in sensory acceptability exist, and elevated concentrations of additives may adversely impact texture and flavor. Further investigations are necessary to optimize formulations that balance consumer preferences with functional improvements.

This study aimed to assess the effects of incorporating plum pomace powder into yogurt at varying concentrations (4%, 6%, and 8%) on the physicochemical, phytochemical, sensory attributes, and color characteristics of the final product.

MATERIALS AND METHODS

One hundred liters of cow's milk were supplied by the Rediu Iași Research Station of the University of Life Sciences. Ten kilograms of perfectly ripe plums (*Prunus domestica* var. *Stanley*) were gathered in September 2024 from the Vasile Adamachi Experimental Educational Station in Iasi, Romania. Following that, the plums were kept at 4°C until they were prepared for processing. The plums were first sorted, then washed with distilled water, cut in half, and the seeds were taken out before being shredded and compressed.

The Bosch MES3500 machine, manufactured by Philips Consumer Lifestyle B.V. in Drachten, Netherlands, was utilized to extract pumpkin juice, resulting in plum pomace (PP). The plum pomace (PP) was kept at –20°C in plastic bags prior to the freeze-drying procedure. The plum pomace (PP) underwent freeze-drying for forty-eight hours at –42°C at a pressure of 0.10 mBar. A freeze-dryer with a moisture content of 6.0% (BIOBASE BK-FD10T, Jinan, China) was used for performing the process. The obtained PP, which had a mean particle diameter of 450 μm, was ground up for 50 seconds in a grinding mill. Subsequently, it was kept in an airtight glass jar at room temperature until analysis. Before the yogurt was introduced, the PP underwent sterilization with a UV lamp in order to destroy contaminants.

Milk collection, sampling, and analysis

From the farm's storage tank, 100 liters of milk were extracted transferred into sanitized containers, where they were stored for twenty-five hours at 4°C. Subsequently, milk had been included into the laboratory's analytical procedures following appropriate homogenization. The physicochemical parameters of milk samples, such as moisture content, total solid, pH, solid non-fat content, protein content, and fat content, were determined according to AOAC protocols (Rațu et al., 2021). The reagents used to perform chemical analyzes of the obtained products (DPPH (2,2-diphenyl1-picrylhydrazyl), gallic acid solution, sodium acetate solution, potassium chloride solution, ethanol, methanol, sodium carbonate, Folin–Ciocalteu reagent, sodium hydroxide, aluminum chloride, sodium carbonate) were purchased from Sigma Aldrich (Schnelldorf, Germany).

Extraction of biological active compounds from PP

The bioactives were extracted from PP using the ultrasound-assisted extraction technique. Thus, 1.0 g of PP were combined with 10 mL of an 80% ethanol solution that had been acidified with citric acid (at a ratio of 7:1, v/v) before being subjected to ultrasound treatment for 25 minutes at 40°C and 37 kHz using a sonication water bath (Elmasonic S 180 H, Elma, Germany). The resulting supernatant was centrifuged for ten minutes at 6500 rpm and 10°C. The PP extract was then utilized to analyze the antioxidant activity of phytochemicals (anthocyanins, polyphenols, and flavonoids).

Antioxidant activity (DPPH)

The procedure outlined by Karaaslan et al. (2011) was followed in order to neutralize the DPPH radical and assess antioxidant activity. A calibration curve was applied, using Trolox as the standard. In a 100 mL volumetric flask, 3.8 mg of DPPH was homogenized with methanol to the appropriate level to create the DPPH stock solution. Next, 3.9 mL of DPPH (A sample) and 100 µL of the sample to be examined (PP extract) were added into a test tube. For the control sample, 3.9 mL of DPPH (A control) and 100 µL of methanol were used. After 90 minutes of resting in the dark, the absorbance was measured at a wavelength of 515 nm. Trolox equivalents (TE)/g d.w. were expressed as µmol of the results. The change in antioxidant capacity was assessed by determining the inhibition (I%) for each sample using the following formula:

$$I(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Total monomeric anthocyanin content

Total monomeric anthocyanin content was measured using a modified pH differential approach used by Stoica et al. (2024). The absorbance of diluted extracts using different buffer solutions at pH 1.0 and 4.5 was measured at 520 nm and 700 nm. The results showed that the cyanidin-3-glucoside (C3G) content was mg/g of dry weight (d.w).

Total polyphenolic content

Horszwald & Andlauer (2011) described the Folin-Ciocâlteu spectrophotometric approach, which was applied. 200 µL of the PP extract, 1 mL of the Folin-Ciocâlteu reagent, and 15.8 mL of distilled water were added into a test tube. Following ten minutes of standing, three milliliters of 20% sodium carbonate were added, and they were then allowed to stand for an hour at room temperature in the dark. The absorbance was measured at a wavelength of 765 nm. The results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g d.w.) based on a gallic acid standard curve.

Total flavonoid content

The technique described by Arnous et al. (2002) was employed to quantify the total flavonoid content in PP extract. Subsequently, 2 mL of distilled water was combined with 0.25 mL of the PP extract and 0.075 mL of 5% sodium nitrite. Following a five-minute waiting period, 0.15 milliliters of 10% aluminum chloride were introduced, and the mixture was let to react for an additional six minutes. Subsequently, 0.5 mL of 1M sodium hydroxide was introduced. The absorbance of the resulting combination was measured immediately at 510 nm. The catechin standard curve has been used to determine the total flavonoid content, expressed as mg catechin equivalents per gram of sample (mg CE/g d.w.).

Texture profile analysis of yogurt

To determine the texture profile, the samples underwent a double penetration test utilizing a Brookfield CT3 Texture Analyzer (Brookfield Ametek, Middleboro, MA, USA) and an acrylic cylinder with a diameter of 38.1 mm. The test parameters included a trigger load of 0.067 N, a load cell capacity of 9.8 N, a target distance of 15 mm, and a penetration speed of 1 mm/s. All measurements were conducted at 20°C in a controlled environment. TexturePro CT V1.5 software was utilized to analyze the deformation-stress response and compute textural qualities such as gumminess, cohesiveness, hardness, and adhesiveness. Each sample underwent three replications.

Preparation of yogurt enhanced with PP

Full-fat milk was used in the technological procedure. The milk underwent pasteurization for 30 minutes at 62°C, was subsequently chilled to 36°C, and then inoculated with certain lactic bacteria. At 42°C, milk was blended with the starter culture YF-L812 (a commercial product from Chr. HANSEN, Denmark), consisting of a

2:1 mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.
The final pH of 4.8 was achieved after fermenting the inoculated milk for approximately 6.5 hours at 42°C. At this stage, 60 g/kg of sterile PP powder was combined with yogurt and subsequently put into pots. The samples were preserved at 4°C and examined immediately after production. Two distinct varieties of yogurt were manufactured. Four yogurt batches were produced from each production batch: the control batch (CB, which included no PP), and three enhanced yogurt batches (BPP1-4%PP, BPP2-6%PP, BPP3-8%PP) (Figure 1).
The techniques recommended by the Association of Official Analytical Chemists (AOAC) were employed to assess the samples' pH, fat, ash, moisture content, and total protein (Usturoi et al., 2017).
Colour analysis
Using the portable colorimeter with illuminator C-MINOLTA ChromaMeter CR-410 (Konica Minolta, Osaka, Japan), the color of the value-added and control yogurts was assessed. L*, a*, and b* were the color parameters that were identified. Each sample was provided in three separate replicates.

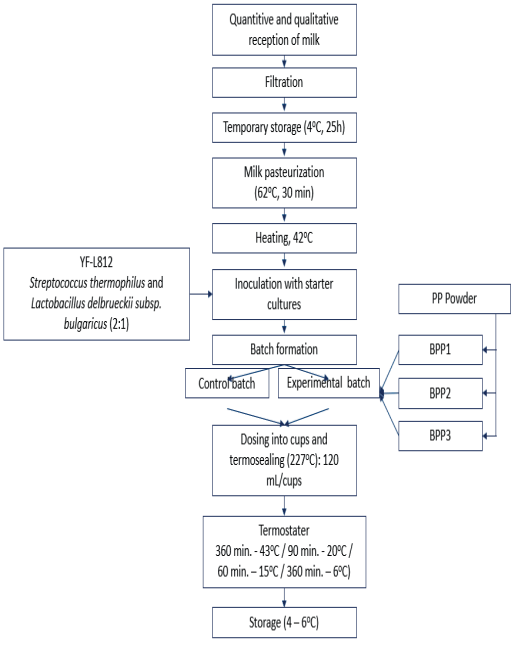


Figure 1. Flow diagram of functional yogurt

Sensorial analysis
A sensory assessment of yogurt samples was conducted by a panel of 16 panellists. The sensory qualities were evaluated using a hedonic scale, where 1 represented the weakest or least pleasant and 9 indicated the strongest or most pleasant. The evaluated qualities included appearance, colour, scent, texture, taste, odour, aftertaste, and overall acceptability. The panellists' ages span from 24 to 50 years, and they were non-smokers. An arbitrary configuration of the samples was used. The overall goal of the study and the procedures for managing personal information were explained to the participants.
Statistical analysis
The data was statistically analyzed using Minitab 19, a statistical processing program, and the data analysis tools package of Microsoft Excel software. Experiments conducted in triplicate were used to compute standard deviations.

RESULTS AND DISCUSSIONS

Spectrophotometric methods were employed to quantify the concentrations of polyphenols, flavonoids, and anthocyanins in ethanolic extracts from PP extract (Table 1). The overall polyphenolic content was 5.11±0.035 mg GAE/g d.w., whereas the total flavonoid content was 1.41±0.014 mg CE/g d.w. A substantial concentration of total anthocyanin content was recorded at 1.44±0.24 mg C3G/g d.w., alongside a notable antioxidant activity of 20.76±0.19 µmol TE/g d.w.

Table 1. Phytochemical characterizations and colorimetric parameters of the PP powder

Parameters	PP Powder
Total anthocyanin content (mg C3G/g d.w.)	1.44±0.24
Total flavonoid content (mg CE/g d.w.)	1.41±0,014
Total polyphenol content (mg GAE/g d.w.)	5.11±0,035
Antioxidant activity (DPPH, µmol TE/g d.w.)	20.77±0,03
Inhibition %	88.16± 0.45
L*	22.346±0.16
a*	10.37±0.05
b*	3.29±0.02

The PP powder was placed in quadrant I (+a*, +b*) based on the colour indices. The projected

values for the L*, a*, and b* parameters are 22.46, 10.27, and 3.29, respectively, based on colour characteristics. Due to its elevated anthocyanin concentration, PP powder exhibited a high a* value, indicating a strong inclination towards red.

Bajić et al. (2020) obtained comparable results in the production of sugar, amidated low-methoxyl pectin, plum pomace lyophilisate, and high-quality plum spread. Textural parameters (maximal force, work of penetration, work of adhesion, intersection time, and intersection force), colour (L*, a*, b*, chroma, and hue angle), and chemical constituents (total phenolic content, total flavonoid content, total monomeric anthocyanins, antioxidant activity) were used to characterize plum spread.

Table 2. Chemical contents values of raw milk

Parameters	Mean
Water (%)	87.17±0.05
Fat (%)	3.85±0.03
Protein (%)	3.35±0.03
Total Solids (%)	12.83±0.07
Solid-non fat (%)	8.98 ±0.06
pH	6.55±0.01

To determine the quality parameters for the raw milk, the main chemical quality indices have been determined. Table 2 presents the chemical composition values of cow's milk samples. The raw milk had a water content of 87.17±0.05% and a total solids content of 12.83±0.07%. The average solid-non-fat content was 8.98±0.06%, with an average fat content of 3.85±0.03%. The average protein level was 3.35±0.03%. The results indicate that each raw milk quality indicator meets the criteria for assessing the milk's overall quality.

Table 3 presents the phytochemical profile and the DPPH free radical scavenging ability of the supplemented and control yogurt. To ascertain the overall phytochemical makeup of the products, an analysis was conducted. Compared to the control batch, the incorporation of PP powder into yoghurt products enhanced phytochemical levels (including anthocyanins, flavonoids, and polyphenols) in a dose-dependent way and improved their ability to scavenge free radicals.

Other authors have similarly documented increases in phytochemical levels. Postolache et al. (2022) demonstrated that increasing the concentration of rhododendron flower powder from 1% to 2% resulted in an increase in anthocyanins, flavonoids, polyphenols, and in vitro antioxidant activity in fortified yogurts, thus demonstrating a dose-dependent impact. Demirkol et al. (2018) found that yogurt infused with grape pomace increased polyphenol content (20 to 52 mg/100 g yogurt) and increased DPPH values. Also, Kennas et al. (2019) documented the beneficial effect of additional fruit by-products on yogurt polyphenols and identified a positive correlation between yogurt polyphenol concentrations and the amount of pomegranate added peel.

The anthocyanin concentration in the PP-supplemented yogurt varieties ranged from 0.73±0.51 mg C3G/100 g d.w. to 1.85±0.019 mg C3G/100 g d.w.

The antioxidant capacity has increased to 20.98±1.19 µmol TE/g d.w. (BPP3) instead of 10.98±1.17 µmol TE/g d.w. for BPP1. Total polyphenolic compounds, total flavonoids, total anthocyanins, and the DPPH free radical scavenging capacity all increased when PP powder was added to the yoghurts' recipe. The antioxidant activity of the improved yoghurts was significantly influenced by the bioactive ingredients derived from PP, resulting in superior values compared to the control samples.

The results in Table 3 validate their value by enhancing the total anthocyanin content and antioxidant activity in yogurt samples included with PP powder. Comparable findings have been documented in additional research concerning the quality evaluation of yogurts. Research by McCullum et al. (2024) indicates that Greek-style yogurt including freeze-dried Illawarra plum powder can enhance its color, total solids, and phytochemical content when refrigerated for up to one month. The study indicates that yogurt can be enhanced by incorporating freeze-dried powder derived from fresh Illawarra plums.

The anthocyanins in the powder provide a uniform, attractive pink colour and contribute to the yoghurt's firmness, so improving its texture. According to Khoo et al. (2017), in addition to serving as natural colorants, several anthocyanin-rich fruit and flowering plants have been extensively utilized in medicine to address different medical conditions. Conversely, plant anthocyanins have been thoroughly investigated

for their therapeutic properties. Anthocyanins exhibit antidiabetic, anticancer, anti-inflammatory, antibacterial, and anti-obesity properties, in addition to preventing heart diseases. Moreover, the fortification does not influence the acidity or syneresis of the yogurt during storage. While the powder enhances levels of anthocyanins, phenolic compounds, and antioxidant activity, further research is required to ascertain the practical benefits of these modifications.

Table 3. The content of phytochemical compounds and the antioxidant activity of added-value yogurt samples

Parameters	Type of yogurts			
	BC	BPP1	BPP2	BPP3
Total anthocyanin content (mg C3G /100 g d.w.)	-	0.73±0.51^{c}	1.14±0.021^{b}	1.85±0.019^{a}
Total flavonoid content (mg CE/g d.w.)	0.72±0.006^{b}	1.12±0.021^{ab}	1.29±0.031^{ab}	1.43±0.007^{a}
Total polyphenol content (mg GAE/g d.w.)	0.98±0.013^{d}	1.62±0.011^{c}	2.31± 0.031^{b}	3.34±0.047^{a}
Antioxidant activity (µmol TE/g d.w.)	2.52±0.25^{d}	10.98±0.12^{c}	16.07±1.13^{b}	20.98±0.14^{a}

Different letters in rows indicate statistically significant differences across the samples (P < 0.05).

Table 4 summarizes the results of the chemical composition analysis of the tested materials. The data analysis indicates significant variations in protein and total solids among yogurts with additional plum pomace powder ($p < 0.05$). The results presented in Table 4 indicate that the protein content of the value-added yogurts with PP powder is significantly higher than that of the control ($p<0.05$). Conversely, the fat content of the value-added yogurts is comparable to that of the control. The ash content exhibited a marginal rise with higher PP powder concentration, rising from 0.95±0.005% in BPP1 to 1.19±0.006% in BPP3.

Comparable results were achieved by Güzeler et al. (2018), who assessed the impact of including dried plums at different concentrations (0%, 6%, 9%, and 12%) on the physicochemical and sensory attributes of probiotic yogurt over a 15-day storage duration. The study indicates that incorporating 12% dried plum into probiotic yogurt initially enhances its physicochemical and sensory attributes; nevertheless, over time, it leads to increased acidity and whey separation. After eight days, the acidic nature of the yogurt resulted in a decline in sensory scores. The findings indicate that 12% dry plum is recommended for probiotic yogurt production due to its favourable balance of flavour, acidity, and functional benefits. Other vegetable by-products have demonstrated an improvement in their antioxidant properties.

Table 4. Chemical composition of added-value yogurts samples

Parameters	Type of yogurts			
	BC	BPP1	BPP2	BPP3
Moisture (%)	87.32±0.031^{a}	84.12±0.039^{b}	81.09±0.041^{c}	79.84±0.045^{d}
Total solid (%)	12.68±0.031^{d}	15.88±0.039^{c}	18.91±0.017^{b}	20.16±0.017^{a}
Fat (%)	3.73±0.017^{b}	4.01±0.015^{a}	4.02±0.015^{a}	4.03±0.012^{a}
Protein (%)	3.65±0.011^{b}	4.12±0.012^{ab}	4.20±0.011^{a}	4.28±0.006^{a}
Ash (%)	0.74±0.009^{d}	0.95±0.005^{c}	1.08±0.004^{b}	1.19±0.006^{a}
pH	4.65±0.004^{a}	4.54±0.009^{b}	4.46±0.011^{c}	4.39±0.006^{d}

Different letters in rows indicate statistically significant differences across the samples (P < 0.05).

Table 5. Color of BC, BPP1, BPP2, and BPP3 yogurts

Parameters	Yogurts type			
	BC	BPP1	BPP2	BPP3
L*	94.51±0.17^{a}	75.66±0.61^{b}	72.74±0.192^{c}	69.09±0.601^{d}
a*	-4.60±0.025^{d}	5.50±0.056^{c}	6.72±0.038^{b}	7.47±0.092^{a}
b*	14.04±0.078^{a}	13.50±0.106^{b}	13.78±0.068^{ab}	13.85±0.107^{ab}

Different letters in rows indicate statistically significant differences across the samples (P < 0.05).

The results of the Lipșa et al. (2024) study underscore the potential utility of onion leaf extract as a source of bioactive compounds with significant antioxidant properties. The study

showed that adding 1% powder to ricotta cheese did not interfere with the commercial LAB starter's ability to ferment the cheese. A bovine whey cheese with distinct functional, physicochemical, and sensory qualities was produced as a result of this enrichment.

According to Stoica et al. (2024), color is a significant sensory attribute that affects how well-liked foods are by consumers. The CIELAB colorimetric parameters were also used to analyze the yogurts. Table 5 presented the results of the color parameters L*, a*, and b* measurements for both the control and PP-supplemented yogurts. The significant rise in the a* value with elevated PP powder content ($p<0.05$) suggests that the bioactive compounds included in the powder have a tendency to assume a red hue. Furthermore, an increase in the concentration of PP powder results in the value-added yogurts displaying diminished degrees of lightness and yellowness, as shown by the L* and b* values. In the analyzed yogurt models, a* increased while L* and b* parameters decreased concurrently with the powder concentration.

Du et al. (2021) observed a comparable tendency by including mulberry pomace, a natural source of pigment, antioxidants, and dietary fiber, into stirred-type functional flavored yogurt.

A nine-point hedonic scale was used to assess the yogurts' sensory qualities. Appearance, color, scent, texture, taste, odor, aftertaste, and general acceptability were the next characteristics listed.

Figure 2 displays the average results obtained from the sensory analysis. Because PP powder contains pigments, particularly anthocyanins, the red color of the resulting yogurts deepened as the concentration of PP powder rose (Figure 2). The BPP2 sample achieved the highest ratings across all nine attributes and maintained an acceptable 6% PP. The panellists noted that the yogurts exhibited a spectrum of hues, with BPP2 displaying the most pronounced red, as the incorporation of PP into the yogurt samples enhanced their red pigmentation. The yogurt samples infused with PP powder were praised for their appealing colour, scent, and odour. The yogurts with additional PP powder were assessed to possess a balanced aftertaste, scent, and taste. The incorporation of PP powder does not significantly influence the fundamental sensory attributes (colour, taste, and scent) regarding consumer approval. The panellists gave all the suggested yogurts favourable reviews. According to the concentration of PP powder used, the produced samples have an appealing colour with red undertones. According to sensory analysis, PP-containing yogurts offer respectable quality attributes and can be well-liked by customers.



Figure 2. Sensory evaluation values of control and added-value yogurts (BC, BPP1, BPP2, BPP3)

Regarding the texture analysis, the configuration of the protein network and the structure of fermented dairy products affect their textural properties. The findings of the texture profile analysis are shown in Table 6. All of the yogurts' textural qualities were considerably impacted by the addition of powder made from pressing plums.

Table 6. Texture of yogurts supplemented with PP after 1, and 14 days

Parameters	Yogurt type	Storage Period (Days)	
		1	14
Hardness, N	**BC**	5.64 ± 0.01^{dA}	5.67 ± 0.01^{dA}
	BPP1	7.52 ± 0.01^{cA}	7.54 ± 0.01^{cA}
	BPP2	9.35 ± 0.01^{bA}	9.38 ± 0.01^{bA}
	BPP3	12.59 ± 0.01^{aA}	12.62 ± 0.01^{aA}
Gumminess, N	**BC**	1.65 ± 0.01^{dA}	1.67 ± 0.01^{dA}
	BPP1	2.84 ± 0.00^{cA}	2.95 ± 0.00^{cA}
	BPP2	4.15 ± 0.02^{bA}	4.23 ± 0.02^{bA}
	BPP3	5.98 ± 0.02^{aA}	6.04 ± 0.02^{aA}
Cohesiveness	**BC**	0.31 ± 0.02^{bA}	0.33 ± 0.02^{bA}
	BPP1	0.35 ± 0.01^{aA}	0.36 ± 0.01^{aA}
	BPP2	0.36 ± 0.01^{aA}	0.38 ± 0.01^{aA}
	BPP3	0.37 ± 0.01^{aA}	0.39 ± 0.01^{aA}
Springiness	**BC**	0.55 ± 0.00^{aA}	0.52 ± 0.00^{aA}
	BPP1	0.32 ± 0.01^{bA}	0.35 ± 0.02^{bA}
	BPP2	0.22 ± 0.02^{cA}	0.27 ± 0.01^{cA}
	BPP3	0.19 ± 0.00^{dA}	0.23 ± 0.00^{cA}
Adhesiveness, mJ	**BC**	-2.51 ± 0.01^{dA}	-2.55 ± 0.01^{dA}
	BPP1	-8.72 ± 0.01^{cA}	-8.76 ± 0.01^{cA}
	BPP2	-11.31 ± 0.03^{bA}	-11.39 ± 0.01^{bA}
	BPP3	-13.65 ± 0.01^{aA}	-13.72 ± 0.02^{dA}

A significant difference ($p < 0.05$) exists between any two means of each sample inside the same column with a different lowercase superscript. A significant difference ($p < 0.05$) exists between any two means of the same sample throughout time inside the same row, indicated by a distinctive superscript capital letter.

The main factor affecting yogurt's texture is its hardness, defined as the force required to induce a specific deformation. The hardness of yogurt supplemented with PP increased with the quantity of powder applied. The dietary fibers in PP absorb more moisture due to its elevated water retention capacity, resulting in increased hardness of the samples. Compared to the control sample, PP-supplemented yogurts demonstrated elevated hardness, gumminess, cohesion, and adhesiveness, alongside reduced elasticity. Over time, every textural attribute of the PP-enriched yogurt increased. The documented results align with previous findings in the field, including the research conducted by Kabir et al. (2020), which utilized banana peels to enhance the storage capacity and bioactive qualities of yogurts.

CONCLUSIONS

Our findings underscore the importance of PP powder as a potent source of bioactive compounds with antioxidant properties. Consequently, the authors suggest including it as a natural component in fortified yogurt.

In comparison to plain yogurt, the PP-supplemented yogurts exhibited elevated total phenolic content and enhanced antioxidant potential. Panellists expressed approval on the improved hue of the yogurt samples, as indicated by a sensory analysis.

The value-added yogurts were considered satisfactory overall. The dairy sector has additional opportunities to satisfy the increasing customer demand for functional meals by producing distinctive, nutritious, and delectable yogurt products. The results obtained confirm the quality of natural components with bioactive potential in plum powders for application in the food sector to develop functional products that promote the circular economy concept.

The results obtained validate the quality of natural components with bioactive potential derived from fruit pomace powders for use in the food sector to produce functional products that promote the circular economy.

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DETERMINATION OF SULFONAMIDE, DAPSONE AND TRIMETHOPRIM RESIDUES IN EGGS BY LC-MS/MS TECHNIQUE

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Abstract

The analysis of sulfonamide and other drug residues is essential to increase consumer confidence in food products and to confirm that the product in question meets all the required conditions both in terms of drug residues and the quality and safety of these products. High-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) was used for the determination of twenty sulfonamides, epson and trypzone sulfonamides. Control hen egg samples were analyzed and any interferences (signals, peaks) were controlled in the region of interest where the analytes under study are expected to elute. Control hen egg samples were then fortified to relevant concentrations with analytes.

Key words*: dapsone, eggs, sulfonamides, trimethoprim.*

INTRODUCTION

Sulfonamides are a category of antibiotics that act specifically on bacteria that cause bacterial infections, therefore they are considered broad-spectrum antibiotics, because they act on several types of bacteria (Grave et al., 2014). Substances such as trimethoprim and dapsone have similar effects to sulfonamides and can be administered together with other pharmacological substances (Sheridan et al., 2008). As for sulfonamides, they have a cumulative effect with trimethoprim and dapsone, but they can also have major negative effects on the human body, producing bacterial resistance, various allergies or carcinogenic effects (Gentili et al., 2005).Protecting consumer health is very important, especially from the point of view of contamination of eggs with sulfonamides, which end up in their content due to treatments carried out for infections in poultry (Wang et al., 2017). The main reactions that can be triggered by their presence in eggs are allergies, acute toxicity and other metabolic and intestinal imbalances (Shao et al., 2005). The consumption of eggs contaminated with certain sulfonamide residues can influence the development of bacterial resistance to antibiotics in the body, being considered a major problem internationally, also affecting the quality of medical treatments (Tian et al., 2013).

Compliance with existing legislation and regulations is very important, so EFSA and Codex Alimentarius have established certain maximum permissible limits (MRLs) for residues of veterinary drugs in products of animal origin (Tölgyesi et al., 2013).

Quality control and traceability in the food chain is an important step due to the determination of sulfonamides through veterinary and safety monitoring programs with the role of improving farm management (Dubreil-Chéneau et al., 2014). Egg quality is influenced by a number of factors, such as: practices used on the farm; drug treatments; hygiene and good practice standards, technology; storage conditions; feed; biological material (Kumar, 2019; Samman et al., 2009; Bologa et al., 2013).The aim of this study was to perform tests for the determination of sulfonamide, dapsone and trimethoprim residues in eggs using the LC-MS/MS technique.

MATERIALS AND METHODS

Samples

The analyzed samples were represented by eggs that were subjected to analysis for the

determination of sulfonamide, trimethoprim and dapsone residues.

Analysis of egg samples

The egg sample is mixed before weighing (2 g eggs) and placed in a 50 ml centrifuge tube. Add a 10 µl internal standard solution (sulfadimidine-13C6, sulfamethoxazole-13C6), with a concentration of 10 µg/ml.

Add 10 ml acetonitrile. Shake the sample until it is homogenized, using the shaker for 15 minutes. Then centrifuge the sample, at a speed of 3500 rpm, for 10 minutes. Prepare the supernatant and repeat the extraction with another 10 ml acetonitrile.

Then combine the extracts and evaporate to dryness, in a nitrogen stream, on a water bath at 50 degrees Celsius.

The residue is reconstituted in 20 ml acetate buffer solution pH 5.3 and shake at maximum speed for 15 minutes. Add 10 ml hexane and shake at maximum speed for 15 minutes. Centrifuge at a speed of 3500 rpm for 10 minutes, separate the organic phase and discard, and apply the aqueous phase to the conditioned SPE cartridges.

Sample purification

For purification, the SPE C18 Strata X column (500 mg/6 ml) was used, as follows: preconditioning with 10 ml methanol and 10 ml 0.2 mol/l acetate buffer, pH 5.3; passing the extract through gravity flow; washing with 10 ml water; drying the column by leaving it under vacuum for 5 minutes; elution with 10 ml acetonitrile.

The eluate is evaporated to dryness on a water bath at 50 degrees Celsius, under a nitrogen stream. The residue is taken up in 1000 µl of 0.1 N HCl. It is shaken for 15 minutes on a shaker at maximum speed and then ultrasonicated for 3 minutes. 30 µl of the egg sample is injected into the LC-MS/MS.

Liquid chromatography coupled with mass spectrometry

Chromatographic column used: Pursuit XRs Ultra C18, 100 x 2 mm/2.8 µm with a flow rate of 0.2 ml/min. Column temperature was 35°C, injection volume 30 µl. Mobile phase (component A: H_2O/ HCOOH 0.1%; component B: Acetonitrile/HCOOH 0.1% gradient A/B).

RESULTS AND DISCUSSIONS

Table 1 shows the analyzed components, the type of measurement and the level.

Table 1. Analyte (measuring) type and level

Compound	Analyte level (µg/kg)
	Chicken eggs (Cmin)
Sulfaguanidines	10*
Sulfacetamides	10*
Sulfonamides	10*
Sulfisomidine	10*
Sulfadiazines	10*
Sulfathiazoles	10*
Sulfapyridine	10*
Sulfamerazine	10*
Sulfamethazine	10*
Sulfamethizole	10*
Sulfamethoxypyridazine	10*
Sulfamonomethoxine	10*
Sulfameter	10*
Sulfachloropyridazine	10*
Sulfadoxine	10*
Sulfamethoxazole	10*
Sulfisoxazole	10*
Sulfabenzamide	10*
Sulfadimethoxine	10*
Sulfaquinoxalines	10*
Sulfaphenazole	10*
Sulfanitran	10*
Trimethoprim	10*
Dapsone	5*
Sulfadimidine - 13C6 (internal standard)	50
Sulfamethoxazole - 13C6 (internal standard)	50

A linear matrix calibration curve was obtained for each analyte of interest. For all substances of interest, correlation coefficients ≥ 0.99 and RSD<15% were obtained, as follows (Table 2).

Table 2. Correlation coefficients

Compound	Chicken eggs	
	Correlation coefficient	RSD (%)
Sulfaguanidines	0.993194	7.518
Sulfacetamides	0.994163	7.442
Sulfonamides	0.996967	7.317
Sulfisomidine	0.998723	6.610
Sulfadiazines	0.996330	10.62
Sulfathiazole	0.996202	6.842
Sulfapyridine	0.998336	6.615
Sulfamerazine	0.998790	6.431

Sulfamethazine	0.999857	2.348
Sulfamethizole	0.995961	7.656
Sulfamethoxypyridaz	0.997556	8.512
Sulfamonomethoxine	0.998534	8.049
Sulfameter	0.997324	8.403
Sulfacloropyridazine	0.996616	5.840
Sulfadoxin	0.998881	6.729
Sulfamethoxazole	0.999129	2.862
Sulfisoxazol	0.999525	6.972
Sulfabenzamide	0.997917	4.789
Sulfadimethoxin	0.996426	11.22
Sulfaquinoxaline	0.999319	10.35
Sulfaphenazole	0.996308	8.693
Sulfanitran	0.997694	7.721
Trimethoprim	0.998464	6.580
Dapsone	0.993902	10.33

For all substances of interest, the correlation coefficients and RSD obtained fall within the established acceptability limits. To establish the selectivity of the analyzed compounds, a chromatographic column of the following type was used:
Pursuit XRs Ultra C18, 100 x 2.0 mm with a particle diameter of 2.8 µm
Since the stationary phase is non-polar C18, and the mobile phase is a mixture of polar compounds (acetonitrile and formic acid 0.1%), the separation mechanism of the sulfonamides is reversed-phase chromatography.
Control samples of chicken eggs were fortified with decreasing concentrations of working standard, starting from the lowest concentration on the calibration curve, up to a concentration that the instrument could no longer detect.
The signal/noise ratio for each compound at a known concentration (Cmin) was calculated using the software (Table 3).

Table 3. The detection limits and quantification limits obtained

Compound	Chicken eggs			
	C_{min} (µg/kg)	S/Z	LOD (µg/kg)	LOQ (µg/kg)
Sulfaguanidines	10*	199	0.151	0.455
Sulfacetamides	10*	200	0.150	0.450
Sulfonamides	10*	15	2.000	6.000
Sulfisomidine	10*	3080	0.010	0.030
Sulfadiazines	10*	1420	0.021	0.063
Sulfathiazoles	10*	427	0.070	0.210
Sulfapyridine	10*	1432	0.021	0.063
Sulfamerazine	10*	1431	0.021	0.063
Sulfamethazine	10*	2104	0.014	0.042
Sulfamethizole	10*	559	0.054	0.161
Sulfamethoxypyridazine	10*	960	0.031	0.093
Sulfamonomethoxine	10*	763	0.039	0.117
Sulfameter	10*	469	0.064	0.192
Sulfachloropyridazine	10*	304	0.099	0.297
Sulfadoxin	10*	2699	0.011	0.033
Sulfamethoxazole	10*	575	0.052	0.156
Sulfisoxazol	10*	865	0.035	0.105
Sulfabenzamide	10*	214	0.140	0.420
Sulfadimethoxin	10*	1402	0.021	0.063
Sulfaquinoxaline	10*	357	0.084	0.252
Sulfaphenazole	10*	666	0.045	0.135
Sulfanitran	10*	17	1.765	5.295
Trimethoprim	10*	2437	0.015	0.045
Dapsone	5*	273	0.055	0.165

For all substances of interest, the detection limits and quantification limits obtained fall within the established acceptability limits.

Recovery
Recovery is the actual percentage of the concentration of a substance recovered during the analytical procedure. It is determined during validation if no certified reference material is available. The percentage recovery obtained for each component is accepted in the range of 80-120 %.
The egg control samples were previously analyzed and no residues of sulfonamides, dapsone and trimethoprim were detected.24 parts of the egg control material were selected and 6 parts were fortified at 0.5 x Cmin; 6 parts at 1 x Cmin; 6 parts at 1.5 x Cmin (minimum concentration) and 6 parts at 2 x Cmin. The samples were analyzed using the same method, the same reagent lots, the same LC-MS/MS equipment, and within a short time frame.The recovery for each compound in each sample was calculated using the following equation:

$$R = \frac{C_{P+Et} - C_P}{C_{Et}} \cdot 100$$

(%) where:
C_{P+Et} - concentration of the compound in the fortified sample;
C_P - concentration of the compound in the sample;
C_{Et} - theoretical concentration of the compound in the fortified sample.

The average recovery was calculated. The percentage recovery obtained for each component is in the range of 80-120% (Table 4).

Table 4. Recovery percentage for chicken eggs

Compound	Chicken eggs		
	Minimum recovery (%)	Average recovery (%)	Maximum recovery (%)
Sulfaguanidines	95.95	101.25	105.04
Sulfacetamides	96.61	100.90	106.54
Sulfonamides	95.08	100.47	104.32
Sulfisomidine	94.82	101.13	107.14
Sulfadiazines	96.32	101.54	107.27
Sulfathiazoles	96.42	100.76	104.53
Sulfapyridine	94.28	99.71	103.99
Sulfamerazine	95.19	99.63	102.61
Sulfamethazine	96.40	100.30	105.14
Sulfamethizole	96.22	101.93	106.24
Sulfamethoxypyridazine	95.48	99.22	102.86
Sulfamonomethoxine	95.76	10021	10385
Sulfameter	95.96	102.28	107.06
Sulfachloropyridazine	98.12	100.75	105.62
Sulfadoxin	95.19	100.26	104.53
Sulfamethoxazol	96.52	100.22	105.22
Sulfisoxazol	95.33	99.81	105.90
Sulfabenzamide	93.86	99.02	103.09
Sulfadimethoxin	96.91	100.46	105.52
Sulfaquinoxaline	95.12	100.67	105.85
Sulfaphenazole	95.49	100.45	104.66
Sulfanitran	96.49	99.97	103.95
Trimethoprim	95.36	101.20	106.22
Dapsone	96.20	100.81	105.08

For all substances of interest, the recovery percentages obtained fall within the established acceptability limits.

Repeatability

From the standard deviation of repeatability sr the "repeatability limit - r" is calculated which allows the analyst to decide whether the difference between repeated analyses of a sample, determined under repeatability conditions, is significant. For each component and for each concentration level it is expected that RSDr ≤ 20%. A set of 24 hen egg samples fortified with the analytes was prepared to produce equivalent concentrations of 0.5; 1; 1.5 and 2 times Cmin (minimum concentration). Six replicates were performed for each assay level. The samples were analyzed and the concentration detected in each sample was determined. The mean concentration, standard deviation, RSDr (%) and repeatability limit were calculated for the results from each series. The samples were analyzed using the same method, the same operator, the same reagent lots, the same LC-MS/MS equipment and within a short time frame. The repeatability limit (r) and RSDr for each series are calculated as follows:

$r = 2.8 \times sr$; $RSDr = sr/Xmed \times 100$ (%)

where:

sr - standard deviation of repeatability;

Xmed - mean concentration.

The experimental results and the calculation results for the mean concentration, standard deviation, RSDr (%) and repeatability limit for the results from each series obtained for hen eggs are as follows (Table 5).

Table 5. Mean concentration, standard deviation, RSDr (%) and repeatability limit for hen eggs

Compound	Chicken eggs				
	Concentration level (µg/kg)	Average concentration (µg/kg)	s_r	RSD_r (%)	r
Sulfaguanidines	5	5.048	0.143	2.84	0.402
	10	10.108	0.260	2.57	0.728
	15	15.242	0.349	2.29	0.979
	20	20.266	0.421	2.08	1.180
Sulfacetamides	5	5.086	0.143	2.82	0.402
	10	10.081	0.233	2.31	0.653
	15	15.125	0.317	2.10	0.887
	20	20.048	0.395	1.97	1.107
Sulfonamides	5	4.984	0.155	3.10	0.433
	10	9.964	0.272	2.73	0.763
	15	15.226	0.321	2.11	0.898
	20	20.214	0.404	2.00	1.132
Sulfisomidine	5	5.100	0.170	3.34	0.477
	10	9.932	0.281	2.83	0.787
	15	15.189	0.382	2.52	1.070
	20	20.390	0.501	2.45	1.401
Sulfadiazines	5	5.034	0.130	2.59	0.365
	10	9.981	0.246	2.47	0.690
	15	15.506	0.339	2.19	0.950
	20	20.460	0.264	1.29	0.740
Sulfathiazoles	5	5.008	0.123	2.46	0.345
	10	9.984	0.212	2.13	0.595
	15	15.298	0.255	1.67	0.715
	20	20.209	0.320	1.58	0.896
Sulfapyridine	5	4.932	0.173	3.51	0.485
	10	9.859	0.275	2.79	0.770
	15	15.004	0355	2.37	0.995
	20	20.315	0.427	2.10	1.196
Sulfamerazine	5	4.976	0.125	2.51	0.349
	10	9.760	0.208	2.13	0.582
	15	15.123	0.284	1.88	0.796
	20	20.116	0.338	1.68	0.947
sulfamethazine	5	4.927	0.140	2.83	0.391
	10	9.940	0.232	2.34	0.651
	15	15.378	0.218	1.42	0.611
	20	20.146	0.216	1.07	0.606
Sulfamethizole	5	5.013	0.153	3.05	0.428
	10	10.031	0.234	2.33	0.656
	15	15.619	0.278	1.78	0.778
	20	20.606	0.353	1.71	0.987
Sulfamethoxypyridazi	5	4.861	0.104	2.13	0.290
	10	9.822	0.191	1.94	0.534
	15	15.126	0.287	1.90	0.803
	20	20.122	0.345	1.71	0.965
Sulfamonomethoxine	5	4.960	0.119	2.40	0.334
	10	9.978	0.215	2.16	0.603
	15	15.236	0.265	1.74	0.741
	20	20.061	0.244	1.22	0.684

Sulfameter	5	5.086	0.145	2.85	0.406
	10	9.882	0.277	2.80	0.776
	15	15.665	0.277	1.77	0.777
	20	20.828	0.301	1.44	0.843
Sulfachloropyridazine	5	5.115	0.112	2.19	0.314
	10	10.091	0.165	1.63	0.461
	15	14.986	0.209	1.40	0.585
	20	19.981	0.247	1.24	0.692
Sulfadoxin	5	4.960	0.142	2.86	0.397
	10	10.008	0.274	2.74	0.768
	15	15.172	0.312	2.05	0.873
	20	20.123	0.323	1.60	0.904
Sulfamethoxazol	5	5.075	0.146	2.87	0.407
	10	10.043	0.232	2.31	0.648
	15	14.886	0.337	2.27	0.945
	20	19.944	0.401	2.01	1.123
Sulfisoxazol	5	4.974	0.174	3.51	0.488
	10	9.872	0.281	2.85	0.788
	15	14.913	0.374	2.51	1.048
	20	20.329	0.393	1.93	1.100
Sulfabenzamide	5	4.948	0.107	2.17	0.301
	10	9.720	0.198	2.04	0.554
	15	14.973	0.290	1.94	0.811
	20	20.021	0.305	1.52	0.855
Sulfadimethoxin	5	5.039	0.150	2.98	0.420
	10	9.967	0.244	2.45	0.682
	15	14.973	0.338	2.26	0.947
	20	20.313	0.368	1.81	1.032
Sulfaquinoxaline	5	5.109	0.178	3.48	0.498
	10	9.876	0.270	2.74	0.757
	15	15.301	0.392	2.56	1.096
	20	19.946	0.370	1.85	1.035
Sulfaphenazole	5	5.103	0.165	3.22	0.461
	10	9.878	0.234	2.37	0.655
	15	14.988	0.329	2.19	0.921
	20	20.214	0.389	1.93	1.090
Sulfanitran	5	5.015	0.132	2.62	0.368
	10	10.050	0.233	2.32	0.654
	15	15.050	0.315	2.09	0.883
	20	19.746	0.353	1.79	0.988
Trimethoprim	5	4.989	0.139	2.79	0.390
	10	10.050	0.262	2.61	0.735
	15	15.449	0.396	2.56	1.108
	20	20.301	0.294	1.45	0.823
Dapsone	2.5	2.511	0.073	2.89	0.203
	5	5.038	0.125	2.48	0.350
	7.5	7.559	0.169	2.23	0.473
	10	10.126	0.221	2.18	0.619

For all substances of interest, the relative standard deviation of repeatability, RSDr (%) obtained falls within the established acceptance limits.

Reproducibility

For each component and for each concentration level, RSDR ≤ 20% is expected. A set of 24 hen egg samples fortified with the analytes was prepared to produce equivalent concentrations of 0.5; 1; 1.5 and 2 times Cmin (minimum concentration).

Six replicates were performed for each assay level. The samples were analyzed and the concentration detected in each sample was determined. The mean concentration, standard deviation, RSD (%) and repeatability limit were calculated for the results in each series.

The samples were analyzed using the same method, by the same analyst, under the same working conditions, on the same equipment, with different reagent lots, on different days.

The first data set is the results of the repeatability test.

The standard deviation of reproducibility (sR), RSDR, RSDHorwitz and the reproducibility limit (R) were calculated for the results of the two sets of samples worked on different days, for each chosen concentration level. The reproducibility limit (R), RSDR and RSDHorwitz for each series are calculated:

$$R = 2.8 \times s_R;\ RSD_R = s_R/X_{med} \times 100\ (\%).$$

$$RSD_{Horwitz} = 2^{(1-0{,}5\log x_{med})}(\%)$$

where: sR - standard deviation of reproducibility; Xmed - mean concentration. The experimental results and calculation results for mean concentration, standard deviation, RSDR (%) and reproducibility limit for the results of each series obtained for hen eggs are as follows (Table 6).

Table 6. Mean concentration, standard deviation, RSDR (%) and reproducibility limit for hen eggs

Compound	Chicken eggs						
	Concentration level (µg/kg)	Average concentration (µg/kg)		S_R	RSD_R (%)	R	RSD_{Horw} (%)
		Series 1	Series 2				
Sulfaguanidine	5	5.048	5.022	0.152	3.02	0.426	1.57
	10	10.108	10.100	0.262	2.60	0.735	1.41
	15	15.242	15.083	0.376	2.48	1.052	1.33
	20	20.266	20.199	0.445	2.20	1.245	1.27
Sulfacetamide	5	5.086	5.046	0.155	3.06	0.434	1.57
	10	10.081	10.048	0.240	2.38	0.671	1.41
	15	15.125	15.245	0.334	2.20	0.935	1.33
	20	20.048	20.045	0.403	2.01	1.129	1.27
Sulfanilamide	5	4.984	5.103	0.170	3.37	0.475	1.57
	10	9.964	9.832	0.290	2.93	0.812	1.42
	15	15.226	14.965	0.378	2.50	1.057	1.33
	20	20.214	20.062	0.454	2.25	1.270	1.27
Sulfisomidin	5	5.100	5.039	0.188	3.71	0.526	1.57
	10	9.932	10.198	0.315	3.13	0.883	1.41
	15	15.189	14.992	0.401	2.66	1.124	1.33
	20	20.390	19.933	0.531	2.63	1.487	1.27
Sulfadiazine	5	5.034	4.940	0.146	2.94	0.410	1.57
	10	9.981	10.035	0.254	2.54	0.712	1.41
	15	15.506	15.190	0.367	2.39	1.028	1.33
	20	20.460	19.824	0.469	2.33	1.314	1.27
Sulfathiazole	5	5.008	4.969	0.146	2.93	0.409	1.57
	10	9.984	10.029	0.228	2.28	0.639	1.41
	15	15.298	15.075	0.280	1.85	0.785	1.33
	20	20.209	20.221	0.325	1.61	0.909	1.27
Sulfapyridine	5	4.932	4.888	0.180	3.68	0.505	1.57
	10	9.859	10.031	0.282	2.84	0.789	1.42
	15	15.004	15.127	0.379	2.51	1.060	1.33
	20	20.315	19.876	0.488	2.43	1.368	1.27
Sulfamerazin	5	4.976	5.042	0.129	2.57	0.360	1.57
	10	9.760	9.830	0.219	2.24	0.614	1.41
	15	15.123	15.158	0.287	1.90	0.803	1.33
	20	20.116	20.329	0.356	1.76	0.998	1.27
Sulfametazina	5	4.927	5.019	0.150	3.01	0.419	1.57
	10	9.940	9.893	0.247	2.49	0.693	1.41
	15	15.378	15.112	0.265	1.74	0.741	1.33
	20	20.146	20.176	0.242	1.20	0.677	1.27
Sulfamethizole	5	5.013	5.113	0.181	3.58	0.507	1.57
	10	10.031	9.940	0.257	2.57	0.718	1.41
	15	15.619	15.201	0.391	2.54	1.096	1.33
	20	20.606	20.083	0.469	2.30	1.313	1.27
Sulfamethoxypyridazine	5	4.861	4.971	0.137	2.79	0.385	1.57
	10	9.822	10.041	0.248	2.50	0.694	1.42
	15	15.126	15.080	0.325	2.15	0.911	1.33

	20	20.122	20.228	0.358	1.78	1.003	1.27
Sulfamonomethoxine	5	4.960	4.996	0.121	2.42	0.338	1.57
	10	9.978	9.984	0.225	2.26	0.631	1.41
	15	15.236	15.138	0.291	1.92	0.816	1.33
	20	20.061	20.181	0.252	1.25	0.707	1.27
Sulfameter	5	5.086	4.929	0.179	3.57	0.501	1.57
	10	9.882	10.100	0.302	3.02	0.846	1.41
	15	15.665	15.031	0.434	2.83	1.215	1.33
	20	20.828	20.193	0.454	2.21	1.270	1.27
Sulfachloropyridazine	5	5.115	5.047	0.114	2.24	0.318	1.57
	10	10.091	10.091	0.169	1.68	0.475	1.41
	15	14.986	15.218	0.243	1.61	0.679	1.33
	20	19.981	19.706	0.297	1.50	0.831	1.27
Sulfadoxin	5	4.960	4.989	0.147	2.96	0.413	1.57
	10	10.008	9.864	0.275	2.77	0.771	1.41
	15	15.172	14.988	0.329	2.18	0.922	1.33
	20	20.123	20.035	0.358	1.78	1.002	1.27
Sulfamethoxazol	5	5.075	5.006	0.152	3.01	0.425	1.57
	10	10.043	10.276	0.269	265	0.754	1.41
	15	14.886	15.121	0.368	2.45	1.030	1.33
	20	19.944	19.966	0.410	2.06	1.149	1.27
Sulfisoxazol	5	4.974	5.061	0.181	3.60	0.506	1.57
	10	9.872	9.986	0.298	3.00	0.835	1.42
	15	14.913	14.747	0.375	2.53	1.050	1.33
	20	20.329	20.164	0.430	2.12	1.204	1.27
Sulfabenzamide	5	4.948	5.020	0.120	2.41	0.337	1.57
	10	9.720	9.980	0.236	2.39	0.661	1.42
	15	14.973	15.188	0.306	2.03	0.856	1.33
	20	20.021	20.096	0.328	1.63	0.918	1.27
Sulfadimethoxin	5	5.039	4.911	0.164	3.30	0.460	1.57
	10	9.967	9.830	0.250	2.53	0.701	1.42
	15	14.973	15.069	0.348	2.32	0.974	1.33
	20	20.313	19.801	0.442	2.20	1.237	1.27
Sulfaquinoxaline	5	5.109	4.987	0.183	3.63	0.513	1.57
	10	9.876	9.966	0.275	2.77	0.770	1.41
	15	15.301	15.010	0.399	2.63	1.118	1.33
	20	19.946	20.104	0.403	2.01	1.128	1.27
Sulfaphenazole	5	5.103	4.992	0.180	3.57	0.504	1.57
	10	9.878	9.857	0.246	2.50	0.690	1.42
	15	14.988	15.080	0.360	2.40	1.008	1.33
	20	20.214	19.869	0.463	2.31	1.296	1.27
Sulfanitran	5	5.015	5.031	0.144	2.86	0.403	1.57
	10	10.050	10.056	0.243	2.41	0.679	1.41
	15	15.050	14.845	0.330	2.21	0.925	1.33
	20	19.746	19.841	0.363	1.83	1.017	1.27
Trimethoprim	5	4.989	5.006	0.141	2.81	0.394	1.57
	10	10.050	10.159	0.279	2.76	0.781	1.41
	15	15.449	15.315	0.402	2.61	1.125	1.33
	20	20.301	19.731	0.435	2.17	1.217	1.27
Dapsone	2.5	2.511	2.524	0.077	3.08	0.217	1.74
	5	5.038	5.025	0.136	2.70	0.380	1.57
	7.5	7.559	7.552	0.180	2.39	0.505	1.48
	10	10.126	10.098	0.232	2.29	0.649	1.41

For all substances of interest, the relative standard deviation of reproducibility, RSDR (%) obtained falls within the established acceptance limits.

Decision limit CCα

The CCα decision limit must be lower than the Cmin (minimum concentration) for dapsone, sulfonamides and trimethoprim. To determine the decision limit for sulfonamides, trimethoprim and dapsone, a set of 8 hen egg samples was prepared, fortified with the analytes, to produce equivalent concentrations of 0.5; 1; 1.5 and 2 times Cmin (minimum concentration). Two replicates were performed for each analysis level. The samples were analyzed and the decision limit was determined by the calibration curve method (α=1%), in accordance with ISO 11843. For the determination of sulfonamide, trimethoprim and dapsone residues, the decision limit was calculated, in accordance with ISO standard 11843, as follows:

$CC_\alpha = c_y + 2.33 \times s_R$,

where: sR - standard deviation of the intralaboratory reproducibility of the intercept; cy - concentration corresponding to the intercept y. Non-compliant samples are samples in which the concentration of sulfonamides, trimethoprim, respectively dapsone found exceeds CCα, for the confirmatory method (Table 7).

Table 7. The values obtained for the decision limit and CCα

Compounds	Chicken eggs
	CC_α (µg/kg)
Sulfaguanidines	0.26
Sulfacetamides	0.67
Sulfonamides	2.06
Sulfisomidine	0.43
Sulfadiazines	1.61
Sulfathiazoles	1.32
Sulfapyridine	2.01
Sulfamerazine	1.02
Sulfamethazine	1.00
Sulfamethizole	0.94
Sulfamethoxypyridazine	1.86
Sulfamonomethoxine	1.73
Sulfameter	1.03
Sulfachloropyridazine	1.03
Sulfadoxin	1.45
Sulfamethoxazol	0.93
Sulfisoxazol	1.72
Sulfabenzamide	1.89
Sulfadimethoxin	2.41
Sulfaquinoxaline	2.87
Sulfaphenazole	2.46
Sulfanitran	7.94
Trimethoprim	3.09
Dapsone	0.82

For all substances of interest, the values obtained for the CCα decision limits fall within the established acceptability limits.

Stability

The stability of the analyte or of the constituents of the matrix sample during storage or analysis must be controlled. A control chart was prepared on 20 readings on the same matrix, which shows the daily development of the values obtained for the compounds of interest, at the same fortification level (Cmin), by the same analyst on 20 different days. The results of the control chart (warning and control limits) are used in internal quality control. Chicken egg samples were fortified with the working standard at the Cmin level (minimum concentration), analyzed, and the results were entered into the control chart. The values obtained must fall within the limit: V(n) = mean ± 2 x SD

Where: mean - arithmetic mean of the series; SD - standard deviation of the series.
The results of the readings for the control sample must fall within the mean ± 2 x SD range, 3 values within the mean ± 3 x SD range, but not consecutive, or 2 consecutive values within the mean ± 3 x SD range and one value above the mean ± 3 x SD are accepted. At 11 consecutive increasing or decreasing values within the mean ± 2 x SD range, the control chart is redrawn, according to ISO 7870-2:2013. There may be situations in which in the analytical series (current samples) that are compared with the ranges in the initially established control chart, recoveries higher than the mean ± 3 x SD are recorded, provided that the values still fall within the accepted recovery range (80-120%).
The results obtained fall within the mean ± 2 x SD range.

Robustness

The analytical method must be tested under different experimental conditions that may include: variations in pH, temperature, source of reagents, centrifugation speed, vortexing time, stability of the derivatization product over time or as a function of temperature. Robustness testing was performed with minor parameter changes: Modification no. 1 (M1) – vortexing time in the extraction stage is changed from 15 minutes to 10 minutes; Modification no. 2 (M2) – changing the number of revolutions per minute of the centrifuge from 4000 rpm to 3500 rpm; Modification no. 3 (M3) – centrifugation time is changed from 10 minutes to 15 minutes; Modification no. 4 (M4) – in the purification phase, when washing the cartridges, the water flow is not allowed to be gravitational but is forced by applying pressure; Modification no. 5 (M5) – the column drying time is changed from 5 minutes to 10 minutes.
A series of chicken egg samples fortified at the Cmin level (minimum concentration) were processed, as follows: - 3 samples without modifying the working parameters (n01, n02, n03), - 5 samples with one working parameter modified (m1, m2, m3, m4, m5), 2 samples with two parameters modified at the same time (m6, m7). The Youden scheme was applied (Tables 8, 9).

Table 8. Testing the method under the influence of some factors

Factor value	Number of determination combinations (sample set)				
	1(n01)	2(n02)	3(n03)	4(m1)	5(m2)
A/a	A	A	A	a	A
B/b	B	B	B	B	b
C/c	C	C	C	C	C
D/d	D	D	D	D	D
E/e	E	E	E	E	E

Table 9. Testing the method under the influence of some factors

Factor value	Number of determination combinations (sample set)				
	6(m3)	7(m4)	8(m5)	9(m6)	10(m7)
A/a	A	A	A	A	A
B/b	B	B	B	b	B
C/c	c	C	C	c	C
D/d	D	d	D	D	d
E/e	E	E	e	E	e

A = readings in fortified samples, worked without changes in parameter M1; B = readings in fortified samples, worked without changes in parameter M2; C = readings in fortified samples, worked without changes in parameter M3; D = readings in fortified samples, worked without changes in parameter M4; E = readings in fortified samples, worked without changes in parameter M5; a = readings in fortified samples, worked with parameter M1 modified; b = readings in fortified samples, worked with parameter M2 modified; c = readings in fortified samples, worked with parameter; M3 modified; d = readings in fortified samples, worked with parameter M4 modified; e = readings in fortified samples, worked with parameter M5 modified.

Three samples with unchanged parameters (3 readings), 5 samples with a single modified parameter and 2 samples with two parameters modified at the same time (7 readings) were processed. The arithmetic mean of the readings from the samples with unchanged parameters and the arithmetic mean of the readings from the samples with modified parameters were calculated (Tables 10-12).

Table 10. Testing the method under the influence of some factors

Sample No.	M1 modifi-cation	M2 modifi-cation	M3 modifi-cation	M4 modifi-cation	M5 modifi-cation
n01	-	-	-	-	-
m1	+	-	-	-	-
m2	-	+	-	-	-
m3	-	-	+	-	-
m4	-	-	-	+	-
m5	-	-	-	-	+
n02	-	-	-	-	-
m6	-	+	+	-	-
n03	-	-	-	-	-
m7	-	-	-	+	+

"+" indicates the working parameters that have been modified. "-" indicates the working parameters that have not been modified.

Table 11. The readings obtained (n01, n02, n03, m1, m2)

Compound	Sample readings				
	n01	n02	n03	m1	m2
Sulfaguanidine	10.078	9.755	10.258	10.084	10.223
Sulfacetamide	9.890	9.672	9.678	9.906	10.301
Sulphanilamide	9.529	10.241	9.983	10.121	9.684
Sulfisomidine	9.622	10.343	9.652	10.105	10.088
Sulfadiazine	10.144	10.096	9.532	9.696	9.677
Sulfathiazole	9.866	10.052	9.463	9.855	9.555
Sulfapyridine	9.683	9.416	9.673	9.885	9.608
Sulfamerazine	9.438	9.694	9.575	9.855	10.133
Sulfamethazine	9.761	9.942	10.160	10.033	10.090
Sulfamethizole	10.019	10.185	10.217	10.062	10.150
Sulfamethoxypyridazine	9.983	10.029	9.769	10.092	10.054
Sulfamonomethoxine	9.930	10.001	9.893	10.083	9.882
Sulfameter	10.136	9.893	10.199	10.170	9.993
Sulfacloropyridazin	9.916	9.592	9.800	9.946	10.034
Sulfadoxine	10.113	9.780	9.964	9.970	9.727
Sulfametoxazol	9.672	10.129	9.751	9.886	9.886
Sulfisoxazol	9.716	10.011	9.935	9.657	9.855
Sulfabenzamide	9.473	9.674	10.048	9.867	9.931
Sulfadimetoxin	10.267	9.781	9.941	9.933	9.864
Sulfaquinoxalin	10.117	9.973	9.853	10.222	10.096
Sulfaphenazol	10.144	9.748	9.830	9.952	10.205
Sulfanitran	10.099	9.557	9.840	10.054	9.572
Trimethoprim	9.794	9.820	10.062	9.901	10.270
Dapsona	5.188	4.856	5.100	5.136	5.013

Table 12. The readings obtained (m3, m4, m5, m6, m7)

Compound	Sample readings				
	m3	m4	m5	m6	m7
Sulfaguanidine	10.229	9.960	10.093	9.932	10.022
Sulphanilamide	9.499	10.754	10.259	10.200	9.797
Sulfisomidine	9.656	10.139	10.089	10.053	9.880
Sulfadiazine	9.488	9.493	9.988	10.076	9.631
Sulfathiazole	9.430	9.861	9.990	10.127	10.097
Sulfapyridine	9.506	10.067	9.537	9.930	9.797
Sulfamerazine	10.225	9.752	9.699	9.712	9.923
Sulfamethazine	10.668	9.997	9.952	9.919	9.924
Sulfamethizole	9.817	10.193	10.389	10.309	9.774
Sulfametoxipiridazin	10.067	9.900	9.758	9.932	9.867
Sulfamonometoxin	10.073	9.760	9.894	10.001	9.895
Sulfameter	10.088	9.976	9.924	9.856	10.286
Sulfacloropyridazin	10.087	10.148	10.172	9.909	10.010
Sulfadoxin	10.023	10.010	9.890	10.288	10.150
Sulfametoxazol	9.980	9.700	9.707	10.021	9.768
Sulfisoxazol	9.826	9.974	9.628	10.298	9.963
Sulfabenzamida	9.935	10.087	9.605	10.063	10.090
Sulfadimetoxin	10.223	9.882	10.052	10.048	10.329
Sulfaquinoxalin	9.817	9.729	10.129	10.063	9.865
Sulfaphenazol	10.062	10.223	9.787	10.055	9.909
Sulfanitran	10.106	10.081	9.994	10.010	10.044
Trimethoprim	10.427	10.448	9.904	9.695	10.004
Dapsona	5.142	4.955	4.975	5.054	4.971

The difference between the average readings of samples with unchanged parameters and the average readings of samples with modified parameters was calculated and then the square of the differences was calculated (Tables 13-18).

$A_A=\sum(A_i)/9;$ $A_a=\sum(a_i)/1;$ $D_a=A_A-A_a=\sum(A_i)/9-\sum(a_i)/1;$ D_a^2

$A_B=\sum(B_i)/8;$ $A_b=\sum(b_i)/2;$ $D_b=A_B-A_b=\sum(B_i)/8-\sum(b_i)/2;$ D_b^2

$A_C=\sum(C_i)/8;$ $A_c=\sum(c_i)/2;$ $D_c=A_C-A_c=\sum(C_i)/8-\sum(c_i)/2;$ D_c^2

$A_D=\sum(D_i)/8;$ $A_d=\sum(d_i)/2;$ $D_d=A_D-A_d=\sum(D_i)/8-\sum(d_i)/2;$ D_d^2

$A_E=\sum(E_i)/8;$ $A_e=\sum(e_i)/2;$ $D_e=A_E-A_e=\sum(E_i)/8-\sum(e_i)/2;$ D_e^2

Table 13. The results obtained for A(A) A(a) D(a) D(a)2

Compound	**A(A)**	**A(a)**	**D(a)**	**D(a)2**
Sulfaguanidine	10.06	10.084	-0.02	0.0005
Sulfacetamide	9.98	9.906	0.07	0.0048
Sulphanilamide	9.99	10.121	-0.13	0.0161
Sulfisomidine	9.95	10.105	-0.16	0.0250
Sulfadiazine	9.79	9.696	0.10	0.0092
Sulfathiazole	9.83	9.855	-0.03	0.0008
Sulfapyridine	9.69	9.885	-0.19	0.0377
Sulfamerazine	9.79	9.855	-0.06	0.0037
Sulfamethazine	10.05	10.033	0.01	0.0002
Sulfametizol	10.12	10.062	0.05	0.0030
Sulfametoxipiridazin	9.93	10.092	-0.16	0.0266
Sulfamonometoxin	9.93	10.083	-0.16	0.0248
Sulfameter	10.04	10.17	-0.13	0.0172
Sulfacloropyridazin	9.96	9.946	0.02	0.0003
Sulfadoxin	9.99	9.97	0.02	0.0006
Sulfametoxazol	9.85	9.886	-0.04	0.0016
Sulfisoxazole	9.91	9.657	0.25	0.0649
Sulfabenzamide	9.88	9.867	0.01	0.0001
Sulfadimethoxine	10.04	9.933	0.11	0.0121
Sulfaquinoxaline	9.96	10.222	-0.26	0.0685
Sulfaphenazole	10.00	9.952	0.04	0.0019
Sulfanitran	9.92	10.054	-0.13	0.0173
Trimethoprim	10.05	9.901	0.15	0.0213
Dapsona	5.03	5.136	-0.11	0.0116

Table 14. The results obtained for A(B) A(b) D(b) D(b)2

Compound	A(B)	A(b)	D(b)	D(b)2
Sulfaguanidine	10.05	10.11	-0.06	0.0031
Sulfacetamide	9.93	10.12	-0.19	0.0343
Sulphanilamide	10.02	9.94	0.08	0.0065
Sulfisomidine	9.94	10.07	-0.13	0.0182
Sulfadiazine	9.76	9.88	-0.12	0.0139
Sulfathiazole	9.83	9.84	-0.01	0.0002
Sulfapyridine	9.70	9.77	-0.07	0.0054
Sulfamerazine	9.77	9.92	-0.15	0.0232
sulfamethazine	10.05	10.00	0.05	0.0025
Sulfamethizole	10.08	10.23	-0.15	0.0218

Sulfamethoxypyridazine	9.93	9.99	-0.06	0.0036
Sulfamonomethoxine	9.94	9.94	0.00	0.0000
Sulfameter	10.08	9.92	0.16	0.0254
Sulfacloropyridazin	9.96	9.97	-0.01	0.0002
Sulfadoxin	9.99	10.01	-0.02	0.0004
Sulfametoxazol	9.82	9.95	-0.13	0.0167
Sulfisoxazol	9.84	10.08	-0.24	0.0565
Sulfabenzamida	9.85	10.00	-0.15	0.0224
Sulfadimetoxin	10.05	9.96	0.10	0.0090
Sulfaquinoxalin	9.96	10.08	-0.12	0.0135
Sulfaphenazol	9.96	10.13	-0.17	0.0300
Sulfanitran	9.97	9.79	0.18	0.0327
Trimethoprim	10.05	9.98	0.06	0.0039
Dapsona	5.04	5.03	0.01	0.0000

Table 15. The results obtained for A(C) A(c) D(c) D(c)2

Compound	A(C)	A(c)	D(c)	D(c)2
Sulfaguanidine	10.08	10.02	0.05	0.0028
Sulfacetamide	9.94	10.08	-0.14	0.0197
Sulphanilamide	10.05	9.85	0.20	0.0386
Sulfisomidine	9.99	9.85	0.14	0.0183
Sulfadiazine	9.78	9.78	0.00	0.0000
Sulfathiazole	9.84	9.78	0.06	0.0041
Sulfapyridine	9.71	9.72	-0.01	0.0001
Sulfamerazine	9.76	9.97	-0.21	0.0440
Sulfamethazine	9.98	10.29	-0.31	0.0968
Sulfamethizole	10.12	10.06	0.06	0.0037
Sulfametoxipiridazin	9.93	10.00	-0.07	0.0046
Sulfamonometoxin	9.92	10.04	-0.12	0.0143
Sulfameter	10.07	9.97	0.10	0.0100
Sulfacloropyridazin	9.95	10.00	-0.05	0.0021
Sulfadoxin	9.95	10.16	-0.20	0.0420
Sulfametoxazol	9.81	10.00	-0.19	0.0354
Sulfisoxazol	9.84	10.06	-0.22	0.0482
Sulfabenzamida	9.85	10.00	-0.15	0.0231
Sulfadimetoxin	10.01	10.14	-0.13	0.0167
Sulfaquinoxalin	10.00	9.94	0.06	0.0034
Sulfaphenazol	9.97	10.06	-0.08	0.0070
Sulfanitran	9.91	10.06	-0.15	0.0234
Trimethoprim	10.03	10.06	-0.04	0.0013
Dapsona	5.02	5.10	-0.07	0.0054

Table 16. The results obtained for A(D) A(d) D(d) D(d)2

Compound	A(D)	A(d)	D(d)	D(d)2
Sulfaguanidine	10.07	10.05	0.02	0.0003
Sulfacetamide	9.96	9.99	-0.03	0.0008
Sulphanilamide	9.94	10.28	-0.34	0.1129
Sulfisomidine	9.95	10.01	-0.06	0.0034
Sulfadiazine	9.84	9.56	0.28	0.0757
Sulfathiazole	9.79	9.98	-0.19	0.0349
Sulfapyridine	9.65	9.93	-0.28	0.0769
Sulfamerazine	9.79	9.84	-0.05	0.0021
Sulfamethazine	10.07	9.96	0.11	0.0111
Sulfamethizole	10.14	9.98	0.16	0.0256
Sulfametoxipiridazin	9.96	9.88	0.08	0.0059
Sulfamonometoxin	9.97	9.83	0.14	0.0202
Sulfameter	10.03	10.13	-0.10	0.0097
Sulfacloropyridazin	9.93	10.08	-0.15	0.0216
Sulfadoxin	9.97	10.08	-0.11	0.0122
Sulfametoxazol	9.88	9.73	0.15	0.0210
Sulfisoxazol	9.87	9.97	-0.10	0.0106
Sulfabenzamida	9.82	10.09	-0.26	0.0697
Sulfadimetoxin	10.01	10.11	-0.09	0.0084
Sulfaquinoxalin	10.03	9.80	0.24	0.0561
Sulfaphenazol	9.97	10.07	-0.09	0.0087
Sulfanitran	9.90	10.06	-0.16	0.0251
Trimethoprim	9.98	10.23	-0.24	0.0585
Dapsona	5.06	4.96	0.09	0.0090

Table 17. The results obtained for A(E), A(e), D(e), D(e)2

Compound	A(E)	A(e)	D(e)	D(e)2
Sulfaguanidine	10.02	10.24	-0.22	0.0490
Sulfacetamide	9.95	10.06	-0.11	0.0124
Sulphanilamide	10.00	10.03	-0.03	0.0007
Sulfisomidine	9.96	9.98	-0.03	0.0007
Sulfadiazine	9.78	9.81	-0.03	0.0012
Sulfathiazole	9.78	10.04	-0.27	0.0715
Sulfapyridine	9.72	9.67	0.05	0.0029
Sulfamerazine	9.80	9.81	-0.01	0.0002
Sulfamethazine	10.07	9.94	0.13	0.0178
Sulfamethizole	10.12	10.08	0.04	0.0014
Sulfamethoxypyridazine	9.98	9.81	0.17	0.0275
Sulfamonomethoxine	9.95	9.89	0.06	0.0034
Sulfameter	10.04	10.11	-0.07	0.0044
Sulfacloropyridazin	9.93	10.09	-0.16	0.0262
Sulfadoxin	9.98	10.02	-0.04	0.0013
Sulfametoxazol	9.88	9.74	0.14	0.0198
Sulfisoxazol	9.91	9.80	0.11	0.0129
Sulfabenzamida	9.88	9.85	0.04	0.0014
Sulfadimetoxin	9.99	10.19	-0.20	0.0393
Sulfaquinoxalin	9.98	10.00	-0.01	0.0002
Sulfaphenazol	10.03	9.85	0.18	0.0322
Sulfanitran	9.91	10.02	-0.10	0.0108
Trimethoprim	10.05	9.95	0.10	0.0096
Dapsona	5.06	4.97	0.08	0.0068

For each compound, the standard deviation of the differences Di (SDi) was calculated with the formula:

$$S_{Di} = \sqrt{2 * \sum(Di^2)/5}$$

Table 18. The results obtained for D(a)2, D(b)2, D(c)2, D(d)2

Compound	D(a)2	D(b)2	D(c)2	D(d)2
Sulfaguanidine	0.0005	0.0031	0.0028	0.0003
Sulfacetamide	0.0048	0.0343	0.0197	0.0008
Sulphanilamide	0.0161	0.0065	0.0386	0.1129
Sulfisomidine	0.0250	0.0182	0.0183	0.0034
Sulfadiazine	0.0092	0.0139	0.0000	0.0757
Sulfathiazole	0.0008	0.0002	0.0041	0.0349
Sulfapyridine	0.0377	0.0054	0.0001	0.0769
Sulfamerazine	0.0037	0.0232	0.0440	0.0021
Sulfamethazine	0.0002	0.0025	0.0968	0.0111
Sulfamethizole	0.0030	0.0218	0.0037	0.0256
Sulfamethoxypyridazine	0.0266	0.0036	0.0046	0.0059
Sulfamonometoxin	0.0248	0.0000	0.0143	0.0202
Sulfameter	0.0172	0.0254	0.0100	0.0097
Sulfacloropyridazin	0.0003	0.0002	0.0021	0.0216
Sulfadoxin	0.0006	0.0004	0.0420	0.0122
Sulfametoxazol	0.0016	0.0167	0.0354	0.0210
Sulfisoxazol	0.0649	0.0565	0.0482	0.0106
Sulfabenzamida	0.0001	0.0224	0.0231	0.0697
Sulfadimetoxin	0.0121	0.0090	0.0167	0.0084
Sulfaquinoxalin	0.0685	0.0135	0.0034	0.0561
Sulfaphenazol	0.0019	0.0300	0.0070	0.0087
Sulfanitran	0.0173	0.0327	0.0234	0.0251
Trimethoprim	0.0213	0.0039	0.0013	0.0585
Dapsona	0.0116	0.0000	0.0054	0.0090

When SDi is significantly lower than the standard deviation of the method performed under reproducible conditions, the conclusion is that these factors, combined, have no effect on the result, even if each factor alone does not indicate a significant influence and that the method is sufficiently robust to the chosen modifications. The results obtained show that the method is sufficiently robust to the chosen modifications, since SDi is significantly lower than the standard deviation of the method performed under intralaboratory reproducible conditions (Table 19).

Table 19. D(e)2, SDi, SR for the analyzed compounds

Compound	D(e)2	S_{Di}	S_R
Sulfaguanidine	0.0490	0.15	0.26
Sulfacetamide	0.0124	0.17	0.24
Sulphanilamide	0.0007	0.26	0.29
Sulfisomidine	0.0007	0.16	0.32
Sulfadiazine	0.0012	0.20	0.25
Sulfathiazole	0.0715	0.21	0.23
Sulfapyridine	0.0029	0.22	0.28
Sulfamerazine	0.0002	0.17	0.22
Sulfamethazine	0.0178	0.23	0.25
Sulfamethizole	0.0014	0.15	0.26
Sulfametoxipiridazin	0.0275	0.17	0.25
Sulfamonometoxin	0.0034	0.16	0.23
Sulfameter	0.0044	0.16	0.30
Sulfacloropyridazin	0.0262	0.14	0.17
Sulfadoxin	0.0013	0.15	0.28
Sulfametoxazol	0.0198	0.19	0.27
Sulfisoxazol	0.0129	0.28	0.30
Sulfabenzamida	0.0014	0.22	0.24
Sulfadimetoxin	0.0393	0.18	0.25
Sulfaquinoxalin	0.0002	0.24	0.28
Sulfaphenazol	0.0322	0.18	0.25
Sulfanitran	0.0108	0.21	0.24
Trimethoprim	0.0096	0.19	0.28
Dapsona	0.0068	0.11	0.14

Table 20. The uncertainty values calculated for each compound

Compound	Chicken eggs			
	C_{min} (µg/kg)	Composite standard uncertainty, u_c (µg/kg)	Uncertainty extended, u_c (k=2) (µg/kg)	Final result (µg/kg)
Sulfaguanidine	10*	0.878	1.756	10 ± 1.756
Sulfacetamide	10*	0.828	1.656	10 ± 1.656
Sulphanilamide	10*	0.849	1.698	10 ± 1.698
Sulfadiazine	10*	0.804	1.608	10 ± 1.608
Sulfadiazine	10*	1.143	2.287	10 ± 2.287
Sulfathiazole	10*	0.786	1.573	10 ± 1.573
Sulfapyridine	10*	0.797	1.595	10 ± 1.595
Sulfamerazine	10*	0.749	1.497	10 ± 1.497
Sulfamethazine	10*	0.455	0.910	10 ± 0.910
Sulfamethizole	10*	0.862	1.724	10 ± 1.724
Sulfametoxipiridazin	10*	0.927	1.854	10 ± 1.854
Sulfamonometoxin	10*	0.882	1.764	10 ± 1.764
Sulfameter	10*	0.953	1.906	10 ± 1.906
Sulfacloropyridazin	10*	0.679	1.357	10 ± 1.357
Sulfadoxin	10*	0.804	1.608	10 ± 1.608
Sulfametoxazol	10*	0.509	1.019	10 ± 1.019
Sulfisoxazol	10*	0.836	1.671	10 ± 1.671
Sulfabenzamida	10*	0.616	1.231	10 ± 1.231
Sulfadimetoxin	10*	1.192	2.384	10 ± 2.384
Sulfaquinoxalin	10*	1.132	2.265	10 ± 2.265
Sulfaphenazol	10*	0.986	1.973	10 ± 1.973
Sulfanitran	10*	0.869	1.738	10 ± 1.738
Trimethoprim	10*	0.782	1.564	10 ± 1.564
Dapsona	5*	0.555	1.111	5 ± 1.111

The expanded measurement uncertainty for a factor k = 2 was calculated according to the Expanded Uncertainty Calculation Sheet – Chromatography, for each compound. The values of the calculated uncertainties for each compound, expressed in relation to the theoretical (added) concentration of the compound in the sample, are presented in the Table 20.

Varenin et al. (2016) uses liquid chromatography in tandem with mass spectrometry for the simultaneous determination of sulfonamides, trimethoprim and dapsone in muscle, egg, milk and honey. Park et al., 2024 uses the LC-MS/MS method with QuEChERS for the simultaneous determination of sulfonamides, trimethoprim, ormethoprim and dapsone in various zootechnical products. Heller et al., 2002 uses the LC-MS-MS method for sulfonamide residues in eggs and reports 5-10 ng/g (ppb) as the confirmation limit for each drug analyzed.

CONCLUSIONS

Given that all proposed performance parameters were achieved, the values obtained fall within the established acceptability requirements, it can be concluded that the method -Determination of sulfonamide, dapsone and trimethoprim residues by LC-MS/MS, can be used for the determination of sulfonamide, dapsone and trimethoprim residues in a liquid chromatography laboratory.

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HEAVY METALS CONTAMINATION OF SHEEP'S MILK: A REVIEW

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Abstract

Contamination with heavy metals and metalloids represents a danger to animal and human health. The most important sources of contamination are attributed to water and soil due to their potential for propagation in the trophic system. The process of contamination with heavy metals is based on a water-soil-plants-animals-human circuit, which is why it is particularly important to carefully monitor all influencing factors. Their presence even in small quantities affects the processes of cellular homeostasis of organisms with direct implications on renal and hepatic functions. The purpose of this article was to present the toxicity of heavy metals in sheep's milk focused on the sources of contamination, the possibilities of metabolism as well as the influence of age and lactation on the accumulation of heavy metals. In order to ensure an optimal level of security, it is recommended to perform periodic analyses of soil, water, feed and carefully monitor the management processes of dairy products.

Key words*: contamination, dairy products, heavy metals, milk quality, toxicity.*

INTRODUCTION

Heavy metals (cadmium, mercury, arsenic, lead, chromium) enter the soil cycle through various anthropogenic activities or natural processes and lead to toxicities of organisms and human beings (Li et al., 2019). Heavy metals have a density 5x higher than water (Kamran et al., 2013). In wastewater there are significant amounts of heavy metals that cannot be removed, subsequently penetrate the soil and interfere with plant roots being consumed by animals/humans and finally are incorporated into the food chain (Kamran et al., 2013). Borys et al. (2006) divide heavy metals into two categories: non-essential elements: cadmium and lead and essential elements: copper and zinc. Heavy metals are divided into two categories: toxic metals (Pb, Cd, As, etc.) and essential metals (Cu, Zn, Mn, Fe, Ni, Cr, etc.) and depending on their origin they are divided into natural and anthropogenic sources (Abdel-Rahman, 2021). High amounts of heavy metals have been observed in industrial areas compared to unpolluted areas (Zhou et al., 2019). The concentration of toxic elements in milk and dairy products is influenced by manufacturing processes and environmental conditions (Anastasio et al., 2006). The mineral composition of milk is correlated with the nutritional status of the animal, the stage of lactation and climate, genetic and environmental factors, reproduction as well as the handling, transport and processing stages after milking (Vahčić et al., 2010; Zamberlin et al., 2012). Even in small quantities, it causes health diseases in humans and animals, and exposure to heavy metals develops various types of cancer, affects the lungs and liver, and bones become more fragile (Vardhan et al., 2019; Singh et al., 2022). The assimilation of these toxic elements takes place in the liver and kidneys of animals. This result leads to the introduction of the risk of heavy metal transmission into the food chain (Wilkinson et al., 2003).

MATERIALS AND METHODS

To present a database for this study, we conducted a systematic search of scientific publications in the Google Scholar, ScienceDirect, Scopus and PubMed databases. The main keywords used were: heavy metals, influencing factors, sheep's milk, contamination, accumulation of heavy metals, presence of

heavy metals in dairy products, possibilities of metabolism.

PRESENCE OF HEAVY METALS IN THE ORGANISM. PATHWAYS OF ENTRY INTO THE ORGANISM. ASSIMILATION, METABOLISM AND TOXICITY

The mechanism of entry of heavy metals into the human body is through inhalation, the gastrointestinal tract or through the skin. They disrupt cellular homeostasis by damaging lipids, enzymes, proteins, DNA membranes, oxidation of amino acid chains and oxidative stress due to the production of free radicals (Witkowska et al., 2021; Jan et al., 2015; Balali-Mood et al., 2021). Pb poisoning generates infertility and neurotoxicity, Hg has harmful effects on nursing mothers and As causes skin diseases and cancer (Kumar et al., 2018). The first target organ of heavy metal toxicity is the kidney, and their effect is dependent on the exposure time, dose and type of heavy metals (Lentini et al., 2017; Budi et al., 2022). The accumulation of heavy metals in the animal body in relatively small percentages (0.09% Cd, 0.06% Hg and 0.03% Pb) increases liver and kidney tissue concentrations by 5 to 20 times (Rehman et al., 2018). In the case of sheep, iron accumulates in the liver and cadmium in the kidneys and liver (Phillips & Tudoreanu, 2011). Another study highlighted the positive relationship between the level of cadmium analyzed in pastures and its presence in sheep milk, the presence of lead was identified in the kidneys, with higher amounts in autumn than in spring (Węglarzy K., 2010). Heavy metal toxicity can be combated by including the intake of minerals, antioxidants and phytochemicals (Kumar et al., 2018).

FACTORS THAT INFLUENCE THE PRESENCE OF HEAVY METALS IN WATER, SOIL AND FEED

Water is an important source of contamination with metalloids and heavy metals through irrigation with wastewater, application of fertilizer to the field or manure, as well as pesticides that disrupt the normal functioning of the human body (Sankhla et al., 2016). The highest values of the health coefficient were identified in agricultural farms irrigated with wastewater and the lowest values were found in water from tube wells (Iqbal et al., 2020). The main parameters that influence the chemical composition and digestibility of cultivated forages are: light, drought, temperature and nutrients in the soil, for example under the influence of low temperatures and low moisture stress are associated with a higher yield of qualitatively superior forages than high temperatures (Martiniello & Teixeira da Silva, 2011). In the case of pastures, the most significant changes in phosphorus, calcium, magnesium, nitrogen and soil organic matter were observed at depths of 0-15 cm and 15-30 cm, and with the decrease in water content from 40 to 10%, calcium, magnesium and potassium concentrations increased (Egeru et al., 2019). Grazing intensity (heavy, medium, light) influences changes in available water retention at intervals of 0-5 cm, soil nitrogen content at the interval of 30-45 cm, soil pH between 5-15 cm and electrical conductivity at all intervals except for the interval of 0-5 cm. Sheep grazing on vegetation contaminated with heavy metals favors the ingestion of Pb from the soil (Smith et al., 2010; Smith et al., 2009). The mineral composition of alfalfa is defined by 3 components (selenium, copper and zinc), the variability of grasses is formed by 4 elements (zinc, potassium, selenium and phosphorus) and silage corn is rich in iron, potassium and manganese concentrations (Marijanusic et al., 2017). The lowest levels of heavy metals were identified in alfalfa (*Medicago sativa* L.), hay, corn (*Zea mays* L.), cereals, silage, purchased mineral supplements and to a lesser extent in soy-based concentrates (Li et al., 2005). The inclusion of multiple plant species improves the qualitative and nutritional value of forage compared to forage monocultures (Bainard et al., 2020). The key to protecting soil health and nutrients is maintaining biodiversity because forage species have different nutrient concentrations and rooting patterns that influence soil biology and soil-plant-animal interactions (Crotty et al., 2015).

HEAVY METALS CONTAMINATION IN DAIRY PRODUCTS

In milk and dairy products, mineral elements are found in different forms: components of organic molecules (proteins, fats, carbohydrates and nucleic acids) or inorganic salts and ions, and the absorption and use of these elements in the body

depends on their chemical form (Šimun et al., 2012). Heavy metal contamination can also occur from metals used in food processing or in the materials with which food products are packaged (Deshwal & Panjagari, 2020). Suhaj & Koreňovská (2008) demonstrated a positive correlation between elemental markers of grazing soil and milk, dairy products from sheep's milk.

The predominant element in the samples analyzed from raw milk, curd, whey, cheese, as well as other cheese varieties before and after salting was Al, followed by Cd and Pb (Toman et al., 2023).

The amounts of Pb, Cd, As and Se in dairy products presented the following values: 0.054 mg/kg milk powder, 0.009 mg/kg whey powder, 1,100 mg/kg and 1,051 mg/kg in cheese assortments and the highest values were identified for Pb.

Mansour et al. (2019) validates the same conclusion obtained in the study conducted, respectively, among the heavy metals monitored in milk and dairy products, lead was the most frequently detected element in a proportion of 66.7%.

It was found that among the species studied (goats, sheep, cows and buffaloes) higher values of Pb and Cd were found in sheep milk (Rahimi, 2013; Najarnezhad et al., 2015).

Regarding the presence of heavy metals in finished products, it has been observed that in curd, soft, brined, smoked and matured cheeses higher values of calcium, magnesium, phosphorus, chromium, zinc, iron and copper are identified (Borys et al., 2006). In cheeses, the elements that influence Pb and Cd concentrations are caused by the manufacturing process, maturation time, fat content and texture (Hashami et al., 2022).

The study conducted by Park (2006) highlights the highest percentage of zinc (415 mg/100 g) compared to the other species. The content of microminerals in sheep's milk is notable for Fe, Mg, Cu and in cow's milk for Zn and Ba. Potassium is the predominant element in cow's milk while Ca is higher in sheep's milk (Spiteri & Attard, 2017).

Higher amounts of Cu are found in cow's milk than in sheep's milk, while the proportion of Zn is higher in goat's milk than in sheep's milk (Gougoulias et al., 2014).

Table 1 shows the average values of heavy metals from various sources of contamination.

Table 1. Heavy metal content from different sources of contamination

Element	Soil (mg/kg)	Water (µg/L)	Feed (silage)(mg/kg)	Milk (µg/L)	Reference
Pb	17.57	0.07	1.38	0.03-10.46	Zhou et al. (2019)
As	7.91	3.58	0.36	0.004-1.53	Zhou et al. (2019)
Cr	39.93	1.80	1.57	0.02-5.01	Zhou et al. (2019)
Cd	0.16	2.04	0.05	0.01-0.27	Zhou et al. (2019)

According to the study conducted by Zhou et al. (2019), the elements with the greatest capacity to transmit heavy metals in soil and feed are lead and chromium, and in water analyses, arsenic illustrates the most significant values, and lead also highlights the highest share in milk.

INFLUENCE OF THE AREA OF ORIGIN OF THE ENVIRONMENT

Organic matter, soil pH and traffic emissions have a direct impact on the accumulation of heavy metals in urban soils (Wang & Zhang, 2018). Cd and Pb levels are correlated with the traffic area considered, so in the case of road sections with a high degree of traffic the concentration of these heavy metals increases predominantly and decreases with distance from high traffic areas (Yan et al., 2013). The following table shows the permissible limits of heavy metals in soil according to the relevant legislation (Table 2).

Table 2. Concentration [mg/kg d.w.] of metals in soil according to Romanian and European legislation

Elements		Mn	Fe	Ni	Cu	Zn	As	Cd	Pb
Ord. 756/1997	Normal value	900.00	3,000.00	20.00	20.00	100.00	5.0	1.00	20.00
	Maximum threshold	2,000.00	4,500.00	200.00	250.00	700.00	25.00	5.00	250.00
	Intervention threshold	4,000.00	7,000.00	500.00	500.00	1,500.00	50.00	10.00	1,000.00
European average value*		650.00	35,100.00	37.30	13.00	52.00	7.03	0.145	22.60

*Geochemical Atlas of Europe cited by Stirbescu et al. (2019)

The table below presents the average values of heavy metals from different locations (Table 3). The most representative values for Cu and Cd are in the study conducted by Levei et al., (2009) while the degree of lead toxicity was the lowest in Iași (Apostoae & Iancu, 2009). The Ni concentration obtained by Apostoae & Iancu

(2009) was lower than that identified by Chira et al. (2014).

Table 3. Heavy metal concentrations in different locations (mg kg^{-1})

Location	Cd	Cu	Cr	Ni	Pb	Zn	Reference
Baia Mare	1.9-25.4	78.3-962	-	-	87.7-9880	109-11500	Levei et al. (2009)
Zlatna	<330	41.0–12000	-	-	32.0–7860	89.0–15600	Levei et al. (2009)
Sibiu	-	29.6–150	-	-	28.6–761	-	Levei et al. (2009)
Baia Mare	2.7	289.5		65.3	847.9	516.6	Chira et al. (2014)
Iași	0.49	45.36	9.28	38.13	27.73	114.78	Apostoae & Iancu (2009)

HEAVY METALS CONTENT BASED ON PERIOD

Póti et al. (2012) investigated how the vegetation period (April-June) influences heavy metal concentrations. It was found that the Cd level increased with the vegetation period while the Cr concentration gradually decreased, the lowest Pb level was identified in April and the highest in May, followed by a decrease. According to the regulations (2005/87/EC) the maximum concentration of heavy metals in green fodder is 30 mg/kg, 1 mg/kg for Cd and 10 mg/kg for Pb at a content of 12%. A positive correlation of Pb was observed between milk and silage, and As in milk was positively correlated with As in water (Zhou et al., 2019). This indicates that heavy metals are transmitted from the source to the raw material, milk. A positive correlation was found between soil and feed in winter and summer seasons for As (Khan et al., 2021). Tahir et al. (2017) demonstrated that the level of heavy metals during a year varies depending on the season and factors such as soil, feed, milk. Thus, the following results were obtained:

Table 4. Heavy metal concentration over a year

Element	Maximum concentration	Minimum concentration
Ni	July-September	January-March
Co	July-September	October-December
Cr	July-September	October-December
Pb	July-September	January-March
Cd	July-September	October-December

Based on the results obtained by Tahir et al. (2017) it can be concluded that the highest values of heavy metals were recorded during the summer, this is due to soil erosion and the supply of contaminated water near the road that reaches the animals' bodies, and is subsequently transmitted to the milk for consumption. Khan et al. (2021) obtained similar results regarding the higher degree of contamination in the winter season than in the summer season for Cd. During the spring, sheep that grazed had high amounts of Pb, Cu, Hg and low levels of Cd and Zn (Kovacik et al., 2017). The absorption capacity of heavy metals from the soil in the feed is as follows: Zn>Cd>Cu>Pb (Miclean et al., 2019). According to ecotoxicological data, heavy metals or metalloids in soil are based on the following structure Se>Tl>Sb>Cd>V>Hg>Ni>Cu>Cr>As>Ba (Vodyanitskii, 2013).

THE INFLUENCE OF AGE ON THE ASSIMILATION OF HEAVY METALS

The assimilation of heavy metals in milk increases as animals age (Najarnezhad et al., 2015) and the toxicity of heavy metals is significantly higher in adult animals than in young animals (García et al., 2011; Rautio et al., 2010). Animals aged <3 years had lower concentrations of Cd (1.40 ± 1.05 mg/ml) and Pb (7.91 ± 3.60 mg/ml) than animals aged >3 years with values of 2.69 ± 1.67 mg/ml for Cd and 11.8 ± 5.71 mg/ml for Pb (Rahimi, 2013). Animals aged 9-10 years had the highest concentrations of cadmium compared to other age groups (Cui et al., 2013). Adult females have a greater capacity to accumulate heavy metals, especially Zn, while in males the predominant metals are Cd (kidneys) and Pb (liver, muscle and kidneys) (García et al., 2011).

THE INFLUENCE OF LACTATION ON THE ASSIMILATION OF HEAVY METALS

In the early and middle stages of lactation, a decrease in the elements Ca, Mg and Zn was observed, as well as an increase detected in the middle and end of the lactation period. At the same time, in the last stage of lactation, the concentration of Se and Fe increased significantly (Pšenková & Toman, 2020). In stage I of late lactation, the element with the most significant values is Cr, which almost doubled its level. The concentration of Pb shows the highest levels in early and late lactation,

respectively average values in mid-lactation. Antunović et al. (2005) demonstrated that the elements Pb and Cd recorded the lowest values on the 2nd day of lactation, followed by a gradual decrease during the experimental period (10, 30 and 60 days) while As had a low concentration at the beginning and at the end of the 60 days it increased significantly. In Lacaune sheep, the content of heavy metals influences the milk profile in lactation as follows: in late lactation, the elements Co, Mn, Mo, Cr and Pb increase and the level of Zn, Cu decreases, respectively in mid-lactation, Cd increases and the concentration of Fe decreases slightly compared to the levels in early and late lactation (Antunović et al., 2023). Several authors have concluded that from the beginning of lactation to the last phase, the level of Zn is in a decreasing trend with values ranging between 8.9 mg/kg of milk at the beginning of lactation and 3.1 mg/kg of milk at the end of lactation (Antunović et al., 2020; Antunović et al., 2023, Pšenkova & Toman 2020). It has been observed that during lactation, the concentration of K decreases and the levels of Mg, Na, Se, Mn, Mo and Cd increase (Antunović, 2016).

CONCLUSIONS

To improve soil-plant-animal interaction, it is proposed to maintain the health of biodiversity, the use of more cultivated species to improve the nutritional characteristics of the feed consumed by animals. Heavy metal toxicity can be combated by including the intake of minerals, antioxidants and phytochemicals. Another way to prevent heavy metal contamination is to pay increased attention to the processing, transport and manufacturing processes. Periodic sampling by responsible organizations is recommended to ensure high security.

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THE INHIBITION OF HETEROCYCLIC AROMATIC AMINE (HAAs) FORMATION AND MODIFICATION OF THE VOLATILE FLAVOR PROFILE IN ROAST BEEF: A REVIEW

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Abstract

Heterocyclic aromatic amines (HAAs) are potential carcinogens that are formed during thermal processing, particularly when meat is exposed to elevated temperatures such as grilling or roasting. The importance of ingredients and marinades, rich in antioxidants properties (like tea, bay leaf, star anise, red chili, oregano), for inhibit the formation of these harmful compounds.Reducing lipid oxidation and Maillard reaction compounds formation, while improving the nutritional quality, safety and sensory preference of grilled meats. The aim of this study was to determine the inhibitory effects of natural and synthetic antioxidants, seeds, spices, fruit, and plant extracts on HAA formation in roast beef meat.

Key words*: antioxidants, beef, carcinogen, flavour, heterocyclic aromatic amines.*

INTRODUCTION

According to data on nitrogen balance, the European Food Safety Authority (EFSA) recommends an average daily protein intake for adults - both men and women - is 0.66 g per kilogram of body weight; for newborns, it can reach up to 1.12 g per kg of body weight (EFSA, 2021). Reference Values suggest that this is the lowest level of consumption needed to satisfy the advised intake for half of the healthy individuals. Heat processing is the most common method for consuming beef and is considered the last component influencesncing the ultimate quality of meat (Tkacz et al., 2021; Gil et al., 2022). Generally, meat products provide particular nutrients in our daily diet (Teng et al., 2023). They are an abundant source of folic acid, vitamins B6 and B12, and proteins in the human body (Kulczyński et al., 2019). Trace elements include iron, magnesium, selenium, copper, zinc, essential amino acids, and lipids (Bohrer, 2017). Primarily mutagenic and cancer-causing heterocyclic aromatic amines (HAAs) are formed in high-temperature processed foods that are high in protein, including meat, fish, and cooked foods (Chen & Xi, 2022; Yang et al., 2021). Maillard reactions and the pyrolysis of proteins and amino acids are responsible for the formation of HAAs (Jing et al., 2021; Teng et al., 2023). Food safety research has focused on preventing the formation of hazardous substances during high-temperature food preparation (Quan et al., 2022). Epidemiological studies have shown that consumption of foods containing HAAs significantly increases the risk of cancer (Cheng et al., 2023; Meurillon et al., 2020).

MATERIALS AND METHODS

For this study, we conducted a systematic search of scientific publications in the Elsevier, ScienceDirect, Scopus, Springer Nature, Web of Science, MDPI and Wiley databases.This review can provide theoretical reference and technical support for developing strategies to effectively inhibit heterocyclic aromatic amines (HAAs) generation and modification of the volatile flavor profile in roast beef meat to the understanding for consumers and relative food processors.

RESULTS AND DISCUSSIONS

HAAs FORMATION

Meat products are rich in carbohydrates, proteins, amino acids, creatine, creatinine, and

other components, which form heterocyclic aromatic amines (Zhang et al., 2025). Consumers appreciate the particular taste of roasted beef acquired during thermal processing, which is considered to be the main process for flavor creation. The Maillard reaction results from the interaction of amino compounds and reducing sugars, which provides food-rich flavors and sensory qualities (Chu et al., 2025).When meat is cooked at high temperatures, the Maillard reaction results in hazardous substances called HAAs, which are affected by various factors (Nadeem et al., 2021). Broasted beef with ascorbic acid, protein oxidation, caramelization, lipid oxidation, and the Maillard reactions are all connected (Oz et al., 2023). Strecker degradation produces pyrazine flavor compounds in the final stage of MR, where heterocyclic chemicals such as heterocyclic aromatic amines are formed (Wang et al., 2023).

CLASSIFICATION OF HAAs

HAAs were initially found on the surface of grilled fish and roast beef (Oz et al., 2023). More than 30 HAAs have been evaluated in all types of heated foods (Gibis, 2016). Heterocyclic aromatic amines (HAAs) of thermal origin are synthesized through the interaction between proteins and free amino acids at temperatures ranging from 100°C to 300°C. In contrast, pyrolytic HAAs are generated at temperatures surpassing 300 °C.. The primary classification of these two types is largely based on the reactions that lead to their formation (Nadeem et al., 2021). Further research focusing on the structure, temperature, and manufacturing methods has classified polar HAAs (IQ type) and non-polar HAAs (non-IQ type) into two primary categories. Polar HAAs are formed from amino acids, creatine, creatinine, carbohydrates at temperatures between 150-250°C, reducing sugars, and other precursors (Puangsombat et al., 2012; Yu et al., 2016; Yao et al., 2023). Nonpolar HAAs are formed from pyridoindole or dipyridoimidazole via the pyrolysis of amino acids at higher temperatures (Karpavičiūtė et al., 2017). In 2025, Mirza Alizadeh et al. divided heterocyclic aromatic amines into two distinct groups: polar (amino-imidazoazarenes) and non-polar (amino-carbolines). The pyridines, quinolines, and quinoxalines, are all polar HAAs. Non-polar HAAs, in Table 1, represent the α-amino-carboline, β-amino-carboline, γ-amino-carboline, and ε-amino-carboline groups.

Table 1. Several of the polar and non-polar heterocyclic aromatic amines, data processed after Mirza Alizadeh et al. (2025)

Group	HAA	Chemical name
Quinolines	IQ	2-Amino-3-methylimidazo[4,5-f]quinolone
	MeIQ	2-Amino-3,4-dimethylimidazo[4,5-f]quinoline
Quinoxalines	IQx	2-Amino-3-methylimidazo[4,5-f]quinaxalines
	MeIQx	2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline
	4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-f]quinaxaline
	7,8-DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline
	4,7,8-TriMeIQx	2-Amino-3,4,7,8-tettramethylimidazo [4,5-f]quinoxaline
Pyridines	PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
	DMIP	Dimethylimidazopyridine
	TMIP	Trimethylimidazopyridine
α-amino-carbolines	AαC	2-Amino-9H-dipyrido[2,3-b]indole
	MeAαC	2-Amino-3-methyl-9H-dipyrido[2,3-b]indole
β-amino-carbolines	NorHarman	9H-Pyrido[3,4-b]indole
	Harman	1-Methyl-9H-pyrido[3,4-b]indole
γ-amino-carbolines	Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
	Trp-P-2	3-Amino-1-methyl-5H-pyrido[4,3-b]indole
δ-amino-carbolines	Glu-P-1	2-Amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole
	Glu-P-2	2-Amino-dipyrido[1,2-a:3',2'-d]imidazole

EFFECTS ON HUMAN HEALTH

Over the past few decades, the formation of heterocyclic aromatic amines at elevated temperatures has been documented, raising concerns about various health hazards, including their carcinogenic potential (Teng et al., 2023). Since 1993, the International Agency for Research on Cancer (IARC) has been involved in the classification of numerous HAAs. Containing "MeIQ, MeIQx, PhIP, AáC, MeAáC, Trp-P-1, Trp-P-2 and Glu-P-2" as

"Group 2B, possible human carcinogens" and a HAA (IQ) as "Group 2A, probably human carcinogen". Studies have shown that HAA exposure increases the risk of lung, pancreatic, breast, colon, colorectal, and stomach cancer (Barzegar et al., 2019; Bellamri et al., 2021; Oz et al., 2023). PhIP, IQ, MeIQx, and MeIQ have been identified by the National Toxicology Program of the US Department of Health and Human Services as expected human carcinogens (Chen et al., 2019). Experiments with Ames *Salmonella typhimurium* have shown that most HAAs have mutagenic potentials (Khan et al., 2024). HAAs have been reported to be over 100 times more dangerous than aflatoxin B1, and more than 2000 times more harmful than benzo[α]pyrene (Huang et al., 2025).

METHODS OF INHIBITING HAAs

The development of strategies to mitigate the effects of HAA exposure has led to research on the modification of cooking methods and the use of seeds, spices, plant extracts, and fruits (Geng et al., 2024; Oz et al., 2023). Natural antioxidant compounds may influence HAA formation by reducing lipid oxidation (Chu et al., 2024). The radical Maillard reaction pathways can be modified by antioxidants, thereby reducing the productivity of the HAA formation reaction (Cao et al., 2020). Extracts from seeds (Khan et al., 2024), reishi mushroom (Guzel et al., 2024), inhibition mechanism of tea polyphenols (Wang et al., 2024), white and oolong tea (Caliskan et al., 2023), ginseng (Yao et al., 2024), citrus peel (Xu et al., 2023), pomegranate peel (Chen et al., 2023), mulberry leaf extract (Xu et al., 2024), and bitter melon extract Momordica Charantia (Gumus et al., 2025) have demonstrated inhibitory effects on the generation of HAA in different types of meat. Antioxidants have been shown to lower the production of HAAs by blocking different pathways of formation. They scavenge free radicals and trap precursors to lower the levels of HAAs in cooked meat (Bulanda et al., 2025). Previous studies have investigated the inhibitory effects of natural and synthetic antioxidants, seeds, spices, fruit, and plant extracts on HAA formation in cooked beef or marinades, several of which are summarized in Table 2.

Table 2. Some of the types of inhibitors and HAAs inhibitions % in cooked beef

Types of Inhibitors	HAAs inhibitions %	Reference
Nutmeg (Seed) Antioxidants	MeIQ 100%	Teng et al. (2023)
Turmeric (Root) Curcumin, Desmethoxycurcumin, Bisdesmethoxycurcumin	IQ, IQx, 7,8 DiMeIQx, PhIP, Harman, Norharman, AαC 75%	Mahmood (2019)
Sichuan pepper (seed) Phenolic compounds	PhIP 82% IQx 61% MeIQx 28% 4,8Di MeIQx 79%	Zeng et al. (2018)
Turmeric and lemongrass (marinate) Phenolic campounds	IQ, PhIP, Harman, Norharman, AαC 94.7%	Sepahpour et al. (2018)
Pomegranate (seed) Tocopherols, phenolic acids (hydroxybenzoic acid)	IQ, 65% PhIP,65% MeIQx, 59% Norharman, 69%	Keskekoglu & Uren (2017)
Hawthorn Phenolic campounds Flavonoids Proanthocyanins	IQx, IQ, MeIQx MeIQ, 4,8 DiMeIQx, 7,8 DiMeIQx, PhIP, Harman, Nor-Harman, MeAαC,TrP2, 20–100%	Tengilimoglu-Metin et al. (2017)
Artichoke (Flower buds) Mono- and di-caffeoylquinic acids, flavonoids	IQx, IQ, MeIQx, AαC MeIQ, 4,8 DiMeIQx, 7,8 DiMeIQx, PhIP, Harman, TrP2, Meαc NorHarman 25-98%	Tengilimoglu-Metin & Kizil (2017)
Apple, elderberry, grape seed, pineapple Proanthocyanidins, phloridzin, and chlorogenic acid	PhIP, MeIQx, 4,8-DiMeIQx 70%	Cheng et al., (2007)
Apple peel powder Polyphenol	PhIP, 83%, MeIQx: 68; 4,8DiMeIQx, 56%	Sabally et al. (2016)
Grape (Seed) Procyanidins polyphenols	MeIQx, 70% PhIP, 90%	Ahn & Grün (2006)
Savory (leaves) Phenolic compounds	PhIP 37.31%	Damašius et al. (2011)
Oregano (leaves) Phenolic compounds	PhIP 43.28%	Damašius et al. (2011)
Rosemary (Leaves) Rosmarinic acid, carnosol, carnosic acid	MeIQx, 91.7% PhIP, 85.3%	Quelhas et al. (2010)
Hibiscus (marinated) Phenolic compounds	MeIQx 50%	Gibis & Weiss (2010)
Hydrocolloids κ-carrageenan	PhIP, 90% MeIQx, 64% 4,8DiMeIQx, 48%	Yang et al. (2021)
Blueberry Phenolic contents	IQx, IQ, MeIQx, AαC, MeIQ, 7,8 DiMeIQx, Harman, TrP2, 91-98%	Gumus & Kizil (2022)
Rosa rugosa tea (RTE) Phenolic compounds	MeAαC, AαC PhIP, Harman, NorHarman, TrP1,TrP2 75%, 46%	Jamali et al. (2016)
Portulaca oleracea L. (PO) Flavonoid	Harman, NorHarman, Glu-P-1 62.39/68.03/73.75%	Jing et al. (2021)
Momordica charantia L. extract (MCE) Antioxidant	PhIP, IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8- DiMeIQx, AαC, 30.08%	Gumus et al. (2025)
Bay leaf, star anise, and red chili (spices) Capsaicin (phenolic)	Harman, 78.08% NorHarman, 64.11%	Zhou et al. (2024)
Bay leaf, star anise, and red chili (spices) Kaempferol (phenolic)	PhIP, 80.87%	Zhou et al. (2024)

INFLUENCE OF THE STORAGE ON HAAs

The general sensory quality of the beef that was stored at 2°C for 5 and 15 days was elevated, while the levels of IQ, MeIQx, MeIQ, Phe-P 1, and AαC were relatively low during the processing (Szterk & Jesionkowska, 2015).

Improving preservation and storage conditions is one strategy to reduce the accumulation of HAAs in beef production (Lai et al., 2023).

METHODE TO QUANTIFICATION AND IDENTIFICATION HAA

Effective sample preparation procedures and instrumental methods applied for the detection and separation of HAAs (usually μg kg −1 or ppb) are required to improve their accuracy and detectability before instrumental analysis (Barzegar et al., 2019; Cao et al., 2020). The most often used method for analyzing HAAs is high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) (Erdoğan & Özdestan-Ocak, 2022; Gumus et al., 2025).

Combining gas chromatography (GC) and enzyme-linked immunosorbent assays (ELISA) with a detector is one way to identify hazardous compounds (Nadeem et al., 2021; Bulanda et al., 2025).

CONCLUSIONS

To reduce the health risks associated with HAA exposure, efforts are being made to change dietary habits and food processing methods.

By employing differential strategies, it is possible to lower the concentration of HAAs in foods cooked at high temperatures, thereby creating food products that are both healthier and safer by preventing the formation of HAAs.

People generally steer clear of meats that have been heavily processed, particularly when these meats are cooked using high-temperature techniques like frying, roasting, or grilling.

Studies should be conducted to investigate the implementation of this strategy, which not only significantly reduces the content of HAAs in meat products, but also protects public health by reducing the intake of harmful carcinogens by consumers in their daily diet.

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CHEMICAL COMPOSITION AND BIOGENIC AMINES IN WILD BOAR MEAT DEPENDING ON STORAGE PERIOD: A REVIEW

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Abstract

The quality of boar meat also depends a lot on the food ingested during the fattening period. Boar meat, compared to domestic pig meat, is richer in protein, lower in lipids and richer in mineral salts. Studies have shown a reduced calorie content of wild boar meat compared to domestically raised pork, a large amount of essential amino acids, vitamin B12, D and mineral salts (iron, zinc, selenium). Since the meat of young boar is more difficult to digest, it is recommended to prepare meat from mature boar. A peculiarity of the biochemical processes in venison is represented by connective tissue proteins, which have a high degree of densification and polymerization, myofibrillar proteins being abundantly represented. The proteins in game meat are resistant to enzymatic denaturation, and that is why it is difficult to mature game meat. The purpose of this study is to characterize the composition of wild boar meat and the biogenic amine content depending on the storage period.

Key words*: biogenic amines, boar meat, fat, protein, quality.*

INTRODUCTION

Food security is currently being addressed more and more intensively both internationally and nationally, being a major concern regarding the quality of food (Feddern et al., 2019).

Game meat is a meat that is increasingly appreciated and sought after by many consumers lately, due to its good chemical composition, the saturated fatty acids present in game meat, the high protein content, low amounts of fat and due to its special organoleptic characteristics (the specific taste and aroma of game meat) (Tomasevic et al., 2018).

Numerous international studies have been conducted over time on the quality of game meat, namely its advantages, disadvantages, storage methods and preparation for consumption (Font-i-Furnols & Guerrero, 2014; Bodnar et al., 2014; Pickering & Lawrence, 2012).

According to data from the specialized literature, biogenic amines (BA) that are taken up through food consumption have no effects on the body if the quantities ingested are small, but if the quantities are larger they can cause various reactions or disorders in the body (Șoimușan et al., 2024; EFSA, 2011).

Biogenic amines can be found in various fermented or matured products, for example: cheeses, fish, meat or in wine products (Barry et al., 2009; Santos et al., 2003; Novella-Rodriguez et al., 2002).

Studies in the specialized literature confirm the existence of these biogenic amines in both animal and plant-based foods intended for human consumption (Capillas-Ruiz & Herrero 2019).

According to their chemical structure, biogenic amines are divided as follows: heterocyclic amines (histamine, tryptamine), aliphatic amines (putrescine, cadaverine) or aromatic amines (tyramine, phenylethyl amine), (Feddern et al., 2019; EFSA, 2011).

According to Ladero et al., 2010, the main source of contamination with biogenic amines is contaminated foods and fermented beverages.

The main biogenic amines that lead to acute toxicity are histamine and tyramine (Halász et al., 1994).

The main reactions produced by the ingestion of high quantities of biogenic amines are the following: headache, emesis, nausea, skin rashes, allergies, fluctuations in blood pressure (Jae-Hyung et al., 2002).

To prevent contamination with biogenic amines, it is necessary to meet the standards for high-quality raw materials for obtaining food products, monitor temperature and humidity parameters and of course comply with good manufacturing process practices (Doeun et al., 2017).
According to Șoimușan et al., 2024, the human body can be affected by the consumption of cheeses that have uneven amounts of biogenic amines inside them, and a much more careful control of the food production stages is recommended.
The process of formation of biogenic amines in food is influenced by a series of intrinsic factors (pH, water activity, microorganisms) and extrinsic factors (storage method and time, storage temperature) (Benkerroum, 2016).
Putrescine, spermidine, spermine and polyamines are commonly found in the cells of living organisms and play physiological roles according to Kalač & Krausová, 2005.

MATERIALS AND METHODS

As part of this review, we conducted a systematic search of scientific publications in the following online databases: Google Academic, Google Scholar, ScienceDirect, PubMed, Scopus, Elsevier, Springer Nature, Web of Science, and Wiley databases.
In this review, we have followed the essential characteristics of biogenic amines and their formation, including the microorganisms involved in the formation of biogenic amines, respectively the effect of storage conditions on the content of biogenic amines and their control methods.

RESULTS AND DISCUSSIONS

THE EXISTENCE OF BIOGENIC AMINES IN FOODS

According to studies, there is a wide range of foods in which biogenic amines may be present, namely: meat, fish, milk, cheese, fruits, vegetables, beer, wine and other products obtained from fish meat (Krizek et al., 2004; Kim et al., 2003; Simon-Sarkadi, 2002; Halász et al., 1994).
Biogenic amines present in different types of food (fish and fish products, meat products, dairy products, vegetables and other plant products) are represented in Table 1 and the daily dose (mg) is reported.
The quality of game meat, according to (Barendse, 2014) is determined by the general quality of the animal, the state of health, the welfare of the game animals as well as the age, breed, anatomy and physiology from the point of view of lifestyle, in addition to all these aspects, the technology of processing game meat also intervenes. In order to prevent high levels of biogenic amines, special attention should be paid to the temperature conditions of the storage process of food intended for human consumption, their handling practices, and the presence of microbial populations (Visciano et al., 2012).
Of course, the specialized literature also touches on some food technological treatments that are essential to avoid contamination with BA, for example, in the case of meat we have the salting process, the baking process, the fermentation process, the maturation process and the marinating process (Chong et al., 2011).
In the case of a study conducted by Hutařová et al., 2014a, biogenic amines in game meat (pheasant, rabbit and deer) were evaluated. Following the study, the sum of biogenic amines for each animal species did not exceed the value allowed by legislation, so the authors conclude the importance of the quality of hygiene and storage measures regarding game meat foods.
One of the potential indicators regarding the safety and quality of a food, in our case game meat, is the level of biogenic amines, so an increased level of BA is automatically associated with an increased level of microbial contamination followed by spoilage processes (Ruiz-Capillas & Jiménez-Colmenero, 2004).
Other measures to prevent food contamination with biogenic amines in food are represented by the cooling and freezing process (Paulsen et al., 2008a).
Limiting microbial growth may include hydrostatic pressures, irradiation processes, controlled atmosphere packaging processes or even the use of food additives (Hutařová et al., 2013).
Biogenic amines are involved in the deterioration of amino acids under high concentrations of microorganisms, with consequences for the human body (Yang et al., 2025).

According to studies a significant increase in the concentration of biogenic amines was observed inside the meat compared to its outside. Histamine and tyramine, present an increased toxic level, compared to spermine, spermidine, putrescein and cadaverine which are not as toxic (Doeun et al., 2017).

MICROORGANISMS INVOLVED IN THE FORMATION OF BIOGENIC AMINES

According to EFSA, 2011, the process of producing biogenic amines requires available amino acids and decarboxylating amino acids synthesized by specialized bacteria.

Strong activity in fermented food can lead to high levels of biogenic amines (Gonzalez et al., 2003).

Regarding the amount of biogenic amines produced, this depends directly on the level of free amino acids present, which in turn depend on the species and on the amount and activity of decarboxylating enzymes (Hutařová et al., 2014a).

The production process of biogenic amines is influenced by several factors, including, according to data from the specialized literature: food storage temperature, duration and method of food storage, food storage conditions, food pH, food salt content, amount of available oxygen, and last but not least, amount of free amino acids (Ruiz-Capillas & Jimenez-Colmenero, 2004). Together, all of these factors mentioned can lead to variable levels of contamination, in terms of biogenic amines (Ruiz-Capillas & Jimenez-Colmenero, 2004).

The toxicological effects of biogenic amines through food consumption are mentioned in numerous specialized studies, such as respiratory, digestive, cardiovascular effects and including neurological disorders (Gardini et al., 2016; Lehane & Olley, 2000).

The bacterial colonies present can influence the production of biogenic amines as fermentation increases (Wang et al., 2025). High levels of certain biogenic amines cause adverse effects on the body, including: migraines, hypertension, tachycardia, acute respiratory failure, heart palpitations, hot flashes, sweating, nausea, skin rashes and other allergies (Capillas-Ruiz et al., 2019; Tofalo et al., 2016).

In a study carried out in Austria on batches of fermented sausages, fish meat, cheese and sauerkraut, the authors suggest tolerable levels of biogenic amines, namely: 6.8 mg per person in the case of adult women and 8.8 mg per person in the case of adult men, the estimated dose per day (Rauscher-Gabernig et al., 2012).

Regarding the exact toxicity limits for biogenic amines, they are very difficult to establish, because the toxic dose is dependent on the effectiveness of the activity of the detoxification mechanism, which can fluctuate depending on the individual (Brink et al., 1990).

Biogenic amines, especially histamine commonly found in foods, are the major cause of skin problems and body allergies (Silla-Santos (1996).

According to Gill (2007), it specifies that the incidence of enteric pathogens in meat from farmed game is positively better than that of field-hunted animals, due to the location of the bullet in the body, the time of evisceration and the temperatures, all of which factors under certain conditions can trigger the development of biogenic amines.

In Table 1, the main biogenic amines are specified with the physiological effects and toxicological effects produced.

Table 1. The main biogenic amines

Biogenic amines	Physiological effects	Toxicological effects
Histamine	neurotransmitter, body temperature regulation, food intake, learning, memory, allergic reactions, immune response, gastric acid secretion, local hormones, cell growth and differentiation	headache, flushing, sweating, nasal discharge, skin rash, dizziness, itching, edema, hives, difficulty swallowing, diarrhea, increased cardiac output, extrasystoles
Tyramine	neurotransmitter, peripheral vasoconstriction, increased cardiac output, increased respiration, increased blood glucose, release of noradrenaline	headaches, migraines, neurological disorders, nausea, vomiting, respiratory disorders, high blood pressure
Putrescine	regulation of gene expression, cell growth and differentiation	increased cardiac output, tachycardia, hypotension, carcinogenic effects

Source: Ladero et al., 2010

EFFECT OF STORAGE CONDITIONS ON THE CONTENT OF BIOGENIC AMINES IN WILD BOAR MEAT

There are numerous factors that influence wild boar meat according to data from the literature, these factors can be triggered during the hunting

process but also after the hunt (Naila et al., 2010).
The concentration of biogenic amines influences the freshness of game meat, in our case wild boar meat (Paulsen et al., 2008b).
According to the study conducted by Hutařová et al., 2014b, good hygienic quality was maintained when wild boar carcasses were stored for a maximum of 14 days at a temperature of 0°C. The authors of the study also confirm a content of biogenic amines in the case of three wild boar samples taken into the study, which exceeded the limit of 5 mg/kg on day 21 of storage of wild boar meat (Hutařová et al., 2014b).
Regarding the temperature of 15°C, it is considered, according to the study conducted by Hutařová et al., 2014b, an inappropriate storage temperature for wild boar meat, because the content of biogenic amines in 2 samples of game meat exceeds the limit of 5 mg/kg on day 7 of storage (Hutařová et al., 2014b). In the case of small game (pheasants or hares), which are hunted with a hunting gun, and the bullets reaching the various tissues and organs of the digestive system, can raise an alarm signal when the game is not eviscerated immediately after the hunting process, but only a few days later, which leads to a major concern regarding the hygienic quality of the game meat (Paulsen et al., 2008b).
In a study, the authors stored tuna meat at two different temperatures and for a storage period of 9 days, as follows: at a temperature of 0°C, tuna meat showed an increase in histamine of 15 ppm, while at a temperature of 22°C, the increase in histamine was 4500 ppm (Du et al., 2002).
In a study Barnes et al., 1973, they showed that the process of storing uneviscerated small game at a temperature of 10°C, for several days, results in a negative impact on carcass quality only when certain organs of the digestive system are perforated, releasing the contents.
In a study, variations in the levels of pathogens (*Escherichia coli,* staphylococci*, Clostridium perfringens, Listeria monocytogenes*) were evaluated for meat from deer, roe deer and wild boar, with a total of 2919 samples collected from commercial units. The results of the study showed that *Clostridium perfringens* was the most common pathogen, and *Listeria monocytogenes* was the least common pathogen in these game samples. The authors also mention that there were no significant differences in contamination between the types of meat and their refrigerated or frozen storage method (Membré et al., 2011).

CONTROL OF BIOGENIC AMINES

The main way to control the concentration of biogenic amines is temperature, and according to data from the specialized literature, emerging methods are also confirmed, including: modified atmosphere packaging, the irradiation process, high hydrostatic pressure, control of microorganisms and the use of food preservatives (Naila et al., 2010).
The process of reducing biogenic amines can be achieved through adequate temperature control, slowing down microbial growth and of course reducing enzymatic activity in meat, data confirmed by numerous studies (Naila et al., 2010; Emborg & Dalgaard, 2008; Balamatsia et al., 2006; Du et al., 2002).
Data from the literature confirm that the formation of biogenic amines can also occur at temperatures below 5°C, so the authors draw attention to the fact that the control of the production and level of biogenic amines should not be based only on temperature control (Emborg & Dalgaard, 2008).
When the presence of biogenic amines is detected in a food product, it is automatically characterized as having poor hygienic quality and as having been obtained through improper food safety practices, so it is important to remember that in order to control the level of biogenic amines, it is necessary to know with certainty what the triggering factors are that lead to the formation of biogenic amines (Hernandez-Jover et al., 1997).
Many studies specify the use of food additives and preservatives that lead to a decrease in the production of biogenic amines, for example, in one study it was shown that sodium sorbate helps extend the shelf life of seafood (Shalini et al., 2001) or meat-based preparations that contain potassium and ascorbic acid showed better values in terms of biogenic amines (Bozkurt & Erkmen, 2004).
Other inhibitory substances with an impact on biogenic amines are: curcumin, capsaicin and piperine, but the effect of these substances

decreases during the cooking process (Shishodia et al., 2005) or as it is also specified ginger, garlic, onion, red pepper or cinnamon (Mah et al., 2009).

Bover-Cid et al., 2000, state that starter cultures used for the fermentation process can delay the formation of biogenic amines, these starter cultures being represented by bacteria that need optimal conditions to have control over preventing the development of biogenic amines. Mbarki et al., 2009, mention that the preservation process using packaging may be an option for delaying the formation of biogenic amines in meat, mainly due to the fact that it restricts the development of microorganisms and enzymes that are the main triggering factors for the production of biogenic amines.

Regarding foods susceptible to containing biogenic amines, we can mention: meat, fish, milk, vegetables, beer, wine, as well as other fish-derived products (Table 2). Among the most frequently encountered and relevant biogenic amines from a food point of view are histamine, tyramine, putrescein, cadaverine and phenyl-ethylamine (Pickering & Lawrence, 2012).

The process of formation of these compounds in food products is significantly influenced by certain intrinsic factors (pH, water activity, type of microorganisms present), as well as by extrinsic factors (storage time and temperature). According to the specialized data obtained to date, histamine and tyramine are considered the most toxic of the biogenic amines (Naila et al., 2010).

Table 2. Biogenic amines in various foods (mg/day)

Biogenic amines in food categories (mg/day)							
		Histamine	Tyramine	Putrescine	Cadaverine	Phenylethylamine	Tryptamine
Alcoholic beverages	Beer	3.6 - 24.2	18.5 - 124.6	6.2 - 41.9	4 - 26.7	0.6 - 4.3	1.3 - 8.6
	Red wine	2.5 - 12.4	1.6 - 8.5	1.9 - 11.5	0.1 - 1.6	<0.1 – 5	-
	White wine	0.1 - 3.9	0.2 - 6.8	0.2 - 6.5	<0.1 - 0.6	<0.1 - 2.3	-
Fish and fish products	Fermented fish	0.3 - 12.6	2 - 90.4	0.6 – 27	0.3 - 12.4	<0.1 - 3.3	0.1 - 2.3
	Fish and other fish products	8.8 - 41.4	4.8 - 13.8	3.8 - 10.9	18.9 - 53.8	1.8 – 5	2.2 - 6.4
Meat products	Fermented sausages	6.4 - 37.1	17.2 - 99.3	14.5 - 83.6	6.7 - 38.5	1.5 - 8.7	1.9 - 10.7
	Other matured meat products	1.4 - 9.9	5.8 - 42.1	5.3 - 38.6	3.3 - 23.9	0.1 - 2.8	<0.1 - 0.3
	Other meat products	0.8 - 1.4	11.4 - 20.1	20.9 - 36.9	4.3 - 7.5	0.2 - 0.3	0.2 - 0.3
Dairy products	Cheese	13 - 32.1	44 - 108.7	14.3 - 35.3	47 - 116.1	1.9 - 4.6	0 - 12.3
	Yogurt	0.3 - 0.8	1.3 - 4.1	0.3 - 0.9	2.6 - 8.2	-	-
	Other dairy products	0.2 - 0.6	0.1 - 0.4	0.3 - 0.9	0.9 - 2.9	-	-
Vegetables and other plant products	Fermented vegetables	0.8 - 27.6	0.8 - 27.3	4.9 - 164.7	0.8 - 28.2	<0.1 - 1.5	-
	Other vegetables	<0.1 - 0.2	1.1 - 7.6	13.4 - 93.6	3.7 - 25.6	<0.1 - 2.8	-

Source: EFSA, 2011

Table 3. Microorganisms involved in the production of biogenic amines

Target food	Amine Biogena	Microorganism
Cheese	Histamina	*Lactobacillus buchneri*
	Tiramina	*Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Entererocccus hirae, Lactobacillus brevis, Lactobacillus curvatus*
	Putresceina	*Enterobacteriaceae (Enterobacter, Serratia, Escherichia, Salmonella, Hafnia, Citrobacter, Klebsiella) Lactobacillus brevis*
	Cadaverina	*Enterobacteriaceae*
Meat	Histamina	*Enterobacteriaceae, Stphylococcus capitis*
	Tiramina	*Staphylococccus carnosus, Staphylococccus xylosus, Staphylococccus epidermidis, Staphylococccus saprophyticus, Lactobacillus brevis, Lactobacillus curvatus, Lactobacillus sakei, Lactobacillus bavaricus, Carnobacterium divergens, Carnobacterium pisicola*
	Putresceina	*Enterobacteriaceae, M. morganii, S. liquefaciens, Pseudomonas, Lb. curvatus, Enterococcus*
	Cadaverina	*Enterobacteriaceae*
Fish	Histamina	*Morganella morganii, Klebsiella pneumonia, Hafnia alvei, Proteus vulgaris, Proteus mirabilis, Enterobacter cloacae, Enterobacter aerogenes, Serratia fonticola, Serratia liquefaciens, Citobacter freundii, Clostridium* spp., *Pseudomonas fluorescens, Pseudomonas putida, Aeromonas* spp., *Pleisomonas shigelloides, Photobacterium* spp.
Wine	Histamina	*Oenococcus oeni, Lactobacillus hilgardii, Pediococcus parvulus*
	Tiramina	*Lactobacillus brevis, Lactobacillus hilgardii, Leuconostoc mesenteroides, Lactobacillus plantarum, Enterococcus faecium*
	Putresceina	*Lactobacillus brevis, Lactobacillus hilgardii, Leuconostoc mesenteroides, Lactobacillus plantarum, O. Oeni, Lb. buchneri, Lactobacillus zeae*
	Cadaverina	*Enterobacteriaceae*

Source: Ladero et al., 2010

Several species susceptible to producing histamine are reported in the literature, for example: *Psychrotolerans morganella, Photobacterium damselae, Photobacterium phosphoreum, Raoultella planticola and Hafnia alvei* (EFSA, 2011; Hoffman & Wiklund, 2006; Bover-Cid & Holzapfel, 1999), (Table 3).

CONCLUSIONS

Game meat is becoming increasingly sought after by consumers due to its compositional and organoleptic characteristics.

Game meat is susceptible, along with other types of meat, to contamination with biogenic amines, given the levels of microorganisms present at different stages of the food chain. Elevated levels of BA can be prevented by following hygiene standards, good handling practices, and observing temperature parameters during the meat handling, delivery, or storage process.

The existence and application of correct verification measures is essential to ensure the correct quality of game meat, with direct responsibility on the personnel involved along the technological chain of handling and processing.

The existence of biogenic amines in game meat has a major role for two reasons: toxicologically and as a quality indicator.

Therefore, we cannot control biogenic amines by simply refrigerating meat, because it is not enough, as we have stated in this review, so other alternative methods must be used.

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QUALITY ASSESSMENT OF SELECTED MEAT PRODUCTS FROM A LEADING ROMANIAN PRODUCER

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Abstract

This study aims to evaluate the quality parameters of selected meat products, specifically Bănățean Salami and Chicken Breast Frankfurters, produced by a meat processing company in Romania. The research includes a comprehensive assessment of the technological, physicochemical, microbiological, and sensory characteristics of these products. Standard laboratory methods were used to determine moisture, protein, fat, salt content, acidity, and microbial safety. Sensory evaluations were also conducted to assess consumer acceptability. The results revealed significant differences in composition and textural attributes between the two products, influenced by their distinct processing methods. Bănățean Salami exhibited a higher fat content and intense aroma, while Chicken Breast Frankfurters had a lower fat percentage and a more delicate texture. The study confirms the compliance of these meat products with national and European quality standards, highlighting the importance of optimizing production techniques for enhanced consumer satisfaction.

Key words*: food quality control, meat processing, meat products, microbiological safety, physicochemical analysis.*

INTRODUCTION

Meat and meat products play a crucial role in human nutrition, providing essential proteins, vitamins, and minerals necessary for a balanced diet. The meat processing industry has evolved significantly to ensure the safety, quality, and sensory appeal of its products (Georgescu & Banu, 2000). Quality assurance in meat production is critical, as it directly impacts consumer health and market competitiveness (Malos & Malos, 2016). Processed meat products have undergone significant improvements in production techniques, ingredient formulation, and preservation methods to meet increasing consumer demand for high-quality, nutritious, and safe food products (Malos & Malos, 2020). The growing awareness of food safety, sustainability, and health concerns has also influenced consumer choices, prompting the industry to explore new methods for improving product quality while maintaining affordability and taste. It is essential to continuously educate consumers about potentially harmful ingredients added to food, encouraging them to read labels carefully to make informed purchasing decisions and prioritize food safety (Posan et al., 2022).

This study focuses on the quality assessment of two widely consumed products: Bănățean Salami, a cured and fermented meat product, and Chicken Breast Frankfurters, a processed poultry-based product. The objective is to evaluate their physicochemical, microbiological, and sensory attributes to determine compliance with quality standards and consumer preferences (Ianițchi et al., 2023). Understanding the differences between these two products will provide insights into the impact of processing techniques on the final product quality and consumer acceptance, helping manufacturers refine their techniques for optimal results (Ianitchi et al., 2014).

MATERIALS AND METHODS

The selected products, Bănățean Salami and Chicken Breast Frankfurters, were analyzed based on their technological production stages and quality parameters. The production processes of both products involve carefully monitored steps to maintain hygiene, consistency, and overall quality. Each stage of the processing chain, from raw material selection to final packaging, was documented to ensure a comprehensive evaluation.

Bănățean Salami is a cured and fermented meat product, requiring high-quality raw materials to ensure a consistent texture and flavor. The production process begins with the selection of pork and beef cuts, which are then trimmed to remove excess connective tissue and fat. The meat is ground to a specific particle size, ensuring uniform distribution of fat and lean content. A precise blend of curing agents, salt, spices, and starter cultures is added to facilitate fermentation and enhance the characteristic taste. These ingredients not only contribute to the taste but also play a crucial role in inhibiting bacterial growth and improving shelf life (Popa & Stănescu, 1995).

After mixing, the meat is stuffed into casings and left to ferment under controlled temperature and humidity conditions. This fermentation stage is crucial for flavor development and preservation. During fermentation, lactic acid bacteria lower the pH, creating an environment unfavorable for pathogenic bacteria while developing a distinct tangy flavor. The duration and conditions of fermentation significantly impact the final taste and texture of the salami. Following fermentation, the salami undergoes a drying and maturation process, which lasts several weeks, allowing for moisture reduction and texture enhancement. Finally, the product is smoked using natural wood smoke to impart its distinctive aroma and color, contributing to its extended shelf life and reinforcing the traditional taste profile associated with fermented meats (Malos & Malos, 2020).

Chicken Breast Frankfurters are finely emulsified meat products that undergo rigorous processing to achieve a smooth and uniform consistency. The process begins with selecting lean chicken breast meat, which is finely ground and mixed with water, salt, stabilizers, and seasonings. Unlike Bănățean Salami, which undergoes fermentation, Chicken Breast Frankfurters rely on thermal processing to ensure safety and uniform texture. Stabilizers and emulsifiers play a crucial role in maintaining consistency and preventing phase separation during cooking.

The finely emulsified meat mixture is then filled into casings and subjected to a two-step thermal treatment: steaming and boiling. This process eliminates potential microbial contaminants while achieving the desired firmness and cohesiveness. Heat treatment plays a critical role in ensuring microbial inactivation and protein gelation, contributing to the final product's structure and stability. After thermal processing, the frankfurters are rapidly cooled to prevent excessive moisture loss and maintain product quality. The final product is vacuum-sealed to enhance shelf stability and maintain freshness during storage and distribution. The combination of thermal treatment and vacuum-sealing helps prevent oxidation and microbial spoilage, extending the shelf life of the product.

The physicochemical composition of the meat products was analyzed using standard laboratory techniques.

Moisture content was determined through oven-drying at 105°C until a constant weight was achieved.

Protein content was measured using the Kjeldahl method, which quantifies nitrogen content and calculates total protein concentration.

Fat content was analyzed via Soxhlet extraction, a method that isolates lipids from the sample. Salt content was measured using Mohr's titration, while acidity was evaluated through titration to assess product stability and shelf life.

A trained panel conducted sensory evaluations to assess key attributes, including color, texture, taste, and aroma. Each product was rated on a standardized scoring system to determine overall consumer acceptability.

The chemical quality indicators of meat products are:

- Composition and nutritional value indicators: total protein content (%), collagen content (%), the collagen/total protein percentage ratio, fat content (%), water content (%);
- Preservation indicators: nitrite content (mg NO_2/100 g product) and NaCl content (%).

For each meat product, 5 samples were collected to undergo organoleptic, microbiological and physicochemical examination. The organoleptic examination consisted of analyzing the organoleptic parameters: shape, appearance, consistency, taste, and smell. The microbiological examination was made to determine *Salmonella*, *Listeria* and *E. coli* presence. The physicochemical examination consisted of determining: moisture, total protein substances, total fat, sodium chloride, nitrites, easily hydrolyzable nitrogen, and the Kreiss reaction.

Technique for the Isolation and Identification of Bacteria from the Genus *Salmonella* Using the Horizontal Method

Stage I: Pre-enrichment in Non-selective Liquid Media. Buffered peptone water is inoculated at room temperature with the sample for analysis, then incubated at 37°C ± 1°C for 18 h ± 2 h.

Stage II: Enrichment in Selective Liquid Media. Rappaport-Vassiliadis soya broth (RVS broth) and Muller-Kauffmann tetrathionate/ novobiocin broth (MKTTn broth) are inoculated with the culture obtained in the buffered peptone water. RVS broth is incubated at 41.5°C ± 1°C for 24 h ± 3 h, and MKTTn broth is incubated at 37°C ± 1°C for 24 h ± 3 h.

Stage III: Isolation and Identification. From the obtained cultures, two selective solid media are inoculated: Xylose lysine deoxycholate agar (XLD agar); Rambach agar (chromogenic medium), recommended for the isolation and identification of Salmonella spp., including lactose-positive strains and the serotypes Salmonella typhi and Salmonella paratyphi, according to the manufacturer's instructions. Both media (XLD and Rambach agar) are incubated at 37°C ± 1°C and examined after 24 h ± 3 h.

Stage IV: Confirmation of Identity. Isolated colonies, presumed to be *Salmonella*, are subjected to confirmation through biochemical and serological tests (Şuler A. et al., 2019).

Procedure for Determining *Listeria*

Pre-enrichment: 25 grams of the sample are mixed with 225 milliliters of Demi-Fraser broth. Incubation is carried out at 30°C for 24 hours.

Selective Enrichment: 1 milliliter of the culture obtained from resuscitation and pre-enrichment is inoculated into 100 milliliters of Demi-Fraser broth. Incubation is carried out at 30°C for 24 hours.

Transfer to Selective Isolation Medium: 0.1 milliliters of the culture, either directly or from a mixture of 0.5 milliliters culture + 4.5 milliliters KOH, is streaked onto the surface of Palcam agar. Incubation is performed at 35°C for 24-48 hours.

Isolation of Characteristic Colonies: Five typical colonies are selected and subcultured on Petri dishes with nutrient agar (or TSYEA agar). Incubation is carried out at 35-37°C for 18-24 hours. Identification and Confirmation are performed using specific biochemical tests (Şuler et al., 2021).

MUG Method for the Rapid Enumeration of *E. coli* in Refrigerated or Frozen Foods

This test is based on the determination of glucuronidase, an enzyme possessed by most *E. coli* strains, unlike other intestinal bacteria. The MUG substrate (4-methylumbelliferyl-β-D-glucuronide) is incorporated into LT broth. Inoculated tubes are incubated under specific conditions and examined under UV light for the presence of a fluorogenic glucuronidase as the final product. Fluorescence in LT-MUG thermolabile medium indicates the presence of *E. coli*.

Determination of moisture content

The water content is determined by the method described in the international standard SR ISO 1442:2010. The method involves heating a certain amount of sample to a heat source at a temperature of 103˚C ± 2˚C until constant weight is achieved, after forming a homogeneous mixture of the sample to be analyzed, sand, and ethanol. The weight loss, calculated as a percentage, represents the water content. It is recommended that the determination be performed in duplicate for each sample taken for analysis.

Apparatus and glassware used: Common laboratory apparatus; Mechanical or electric homogenizer capable of homogenizing the sample for analysis (This includes a high-speed rotating knife or a meat grinder with a sieve whose hole diameter does not exceed 4.0 mm); Weighing flasks with lids made of porcelain or metal (e.g., nickel, aluminum, stainless steel) with a diameter of at least 60 mm and a height of approximately 40 mm; Glass rod; Drying oven, electrically heated, capable of being maintained at a temperature of 103˚C ± 2˚C; Desiccator containing an effective drying agent, such as silica gel; Analytical balance, capable of weighing with an accuracy of ± 0.001 g; clean sand, acid-washed, with a granulation such that it can pass through a sieve with a hole size of 1.4 mm but not through a sieve with a hole size of 250 μm. The sand, before use, is dried at a temperature of 150-160ºC and stored in a tightly sealed bottle.

It is important to take a truly representative sample that has not been damaged or altered during transport or storage. The laboratory sample mass must not be less than 200 g. The laboratory sample is homogenized using appropriate equipment. If a meat grinder is used, the sample is passed through the grinder at least twice. The homogenized sample is placed in an appropriate container, sealed tightly, and filled completely. The container is closed and stored in such a way as to prevent degradation or changes in its composition. Care must be taken to ensure that the sample temperature does not exceed 25ºC. The sample should be analyzed as quickly as possible, but always within 24 hours of homogenization. Transfer a quantity of sand, three or four times the mass of the sample being worked with, into a weighing flask along with a glass rod, and place it in the drying oven for 30 minutes at a temperature of 103ºC. The flask, along with the contents and rod, is then cooled to room temperature in a desiccator and weighed using the analytical balance with an accuracy of 0.001 g (m_0). Weigh a sample with a mass between 5 g and 8 g, in an empty weighing flask along with sand and the glass rod with an accuracy of 0.001 g (m_1). A homogeneous mixture of the sample and sand is formed with the help of the glass rod. If difficulties arise in homogenizing the sample, ethanol may be added. In this case, the ethanol must evaporate before the sample is placed in the drying oven. Heat the flask containing the sample and glass rod for 2 hours in the oven at a temperature of 103ºC. After the specified time, remove the flask from the oven and place it in a desiccator to cool to room temperature, then weigh it with an accuracy of 0.001 g. The heating, cooling, and weighing operations are repeated until the results of two consecutive weighings, separated by one hour of heating, do not differ by more than 0.1% of the sample mass for analysis (m_2). The moisture content of the sample is calculated using the following formula:

$$w\ \% = \frac{m_1 - m_2}{m_1 - m_0} \times 100$$

where:

m_0 is mass of the flask with the glass rod and sand, in grams;

m_1 – mass of the flask with the glass rod, sand, and sample, before drying, in grams;

m_2 – mass of the flask with the glass rod, sand, and sample, after drying, in grams.

The result is rounded to one decimal place.

Determination of total fat (Soxhlet)

The determination of free fat content in meat and meat products is carried out using the standard SR ISO 1444:2008.

Free fat substances are extracted until exhaustion from the dried sample using an organic solvent (n-hexane or petroleum ether) with the Soxhlet apparatus. After the solvent is removed by evaporation, the extract is dried and weighed, and the result is expressed as a percentage. The water content is removed by the method described in the international standard SR ISO 1442:2010.

Apparatus and glassware used: Homogenization equipment, mechanical or electric, capable of homogenizing the sample for analysis. This includes a high-speed rotating knife or a meat grinder with a sieve with hole diameters of up to 4.5 mm; Extraction cartridge, made of filter paper and degreased; Degreased beaker; Continuous or semi-continuous extraction apparatus, e.g., Soxhlet type; Sand bath or water bath, electrically heated, or a similar appropriate apparatus; Electric drying oven, capable of being maintained at 103 ± 2˚C; Desiccator containing an effective drying agent, such as silica gel; Analytical balance, capable of weighing with an accuracy of ± 0.001 g;

Common laboratory glassware

Reagents: Extraction solvent, n-hexane or alternatively petroleum ether with a distillation range between 40˚C and 60˚C, having a bromine index of less than 1. For both solvents, the residue upon complete evaporation should not exceed 0.002 g/100 mL. Porcelain pieces for boiling.

It is important to take a truly representative sample that has not been damaged or altered during transport or storage. The laboratory sample mass must not be less than 200 g. The sample should be stored in such a way as to prevent degradation or modification of its composition. The sample is homogenized for analysis using appropriate equipment. Care should be taken to ensure that the sample temperature does not exceed 25˚C. If a meat grinder is used, the sample should be passed

through it at least twice. A suitable airtight container is filled with the prepared sample. The container is sealed and stored to prevent degradation and alteration of its composition. The sample should be analyzed as soon as possible, but always within 24 hours of homogenization. A known mass of 5 g to 8 g, weighed with an accuracy of 0.001 g (m_0) from the prepared sample, is dried using the method described in the international standard SR ISO 1442:2010. If desired, the dried sample from the moisture content determination can be used for the free fat determination. For reliable measurements, the minimum fat content present in the sample for analysis must be 0.05 g. The flask of the extraction apparatus, containing some porcelain pieces for boiling, is dried for one hour in the drying oven set at 103˚C. The flask is allowed to cool to room temperature in a desiccator and weighed with an accuracy of 0.001 g (m_1). The last traces of the dried sample from the drying flask are transferred using cotton wool moistened with extraction solvent, and the cotton wool is also transferred to the cartridge. The cartridge is placed in the extractor of the apparatus. Extraction solvent is poured into the flask of the extraction apparatus; the amount of solvent should be at least one and a half to two times the capacity of the apparatus extractor. The flask is attached to the extraction apparatus. The flask is heated for at least 6 hours in a sand bath or water bath, according to the extraction rate of the apparatus used. After extraction, the flask containing the liquid is removed from the extraction apparatus, and the solvent is removed by distillation using, for example, a sand bath or water bath. The last traces of solvent are evaporated by blowing air if desired. The flask is dried for 1 hour in the drying oven set at 103˚C, and after cooling to room temperature in a desiccator, it is weighed with an accuracy of 0.001 g. The heating, cooling, and weighing operations are repeated until the results of two consecutive weighings, separated by one hour of heating, do not differ by more than 0.1% of the sample mass for analysis (m1). The completion of the extraction is verified by taking a second extraction flask and extracting for another hour with a fresh portion of solvent. The increase in mass should not exceed 0.1% of the mass of the sample for analysis. The free fat content (w_f), as a percentage by mass, is calculated using the following equation:

$$w_f = \frac{m_2 - m_1}{m_0} \times 100\%$$

where:
m_0 is mass of the sample for analysis taken for drying, in grams;
m_1 – mass of the extraction flask with porcelain pieces for boiling, in grams;
m_2 – mass of the flask and porcelain pieces with fat after drying, in grams. The result is reported rounded to one decimal place.

Determination of total protein substances (Kjeldhal method)

The Kjeldahl method is used for determining the nitrogen content (which represents total protein substances) in meat and meat products. It is based on digesting the sample in a concentrated acidic medium, which converts organic nitrogen into ammonium ions. Afterward, ammonia is distilled using a boric acid solution and titrated with hydrochloric acid to calculate the nitrogen content in the sample.

Equipment and Glassware Used: Mechanical meat grinder with a sieve with holes no larger than 4 mm; Sulfurized paper (9 cm x 6 cm); 50 ml burette; Kjeldahl flasks (maximum 800 ml) with a piriform glass stopper, if necessary; Distillation apparatus or vapor distillation apparatus; Heating device; Vapor extraction device for acid vapors; Analytical balance; General laboratory glassware (beakers, graduated flasks, graduated cylinders, pipettes, burettes).

All reagents must be of recognized analytical grade: Sulfuric acid (H_2SO_4): density 1.84 g/ml; Copper sulfate ($CuSO_4 \cdot 5H_2O$); Potassium sulfate (K_2SO_4) anhydrous; Sodium hydroxide (NaOH), decarbonated solution with approximately 33 g NaOH per 100 ml of solution; Boric acid (H_3BO_3), solution with 40 g per liter of water; Hydrochloric acid (HCl) 0.1 N; Indicator solution: mixed methyl red and methylene blue; Boiling regulators: glass beads, silica carbide, or porcelain fragments.

Start with a representative sample of at least 200 g. Homogenize the sample by passing it through the meat grinder at least twice. Store the homogenized sample in an airtight container and analyze it within 24 hours. Add some boiling regulators into the Kjeldahl flask. Add 15 g of

anhydrous potassium sulfate and 0.5 g of copper sulfate (II). Weigh 2 g (or 1.5 g for fatty samples) of the sample on sulfurized paper.
Add 25 ml of concentrated sulfuric acid (H_2SO_4) to the Kjeldahl flask, mix gently, and heat slowly at a 40° angle to the vertical until the liquid becomes clear and has a blue-green tint.
The digestion should last at least 2 hours, ensuring that no sulfuric acid is lost due to overheating.
After cooling the flask, add water and transfer the solution into a conical flask with 50 ml of boric acid solution and a few drops of indicator solution. Add 100 ml of NaOH and distill the ammonia. Collect at least 150 ml of distillate.
Titrate the distillate with 0.1 N hydrochloric acid solution using a burette. Record the volume of acid required for the titration. Perform two determinations on the same sample and a control sample.
The nitrogen content is expressed as a mass percentage using the formula:

$$X_{NH3} = 0.0014 \times (V_1 - V_0) \times 100/m$$

where:
V_1 is the volume of 0.1 N HCl used for the determination of the sample;
V_0 – the volume of 0.1 N HCl used for the control sample;
m – the mass of the sample being tested (in grams).
To express the result as total protein, multiply the total nitrogen value by a factor of 6.25.

Quantitative determination of easily hydrolyzable nitrogen (titration with HCl)
The method is based on the fact that easily hydrolyzable nitrogen, released with magnesium oxide in the form of ammonia, is distilled with water vapor and captured in a boric acid solution, which is then dosed by titration with hydrochloric acid.
Reagents: Perchloric acid solutions: 6 g/100 ml; Caustic soda solutions: 20 g/100 ml; Standard HCl solutions: 0.05 mol; Boric acid solutions: 3 g/100 ml; Silicone antifoam agent; Phenolphthalein solution: 1 g/100 ml ethyl alcohol; Indicator solution (Tashiro Mixed Indicator): Dissolve 2 g methyl red and 1 g methylene blue in 1000 ml of 95% ethanol.
When handling perchloric acid, necessary safety precautions should be taken as for other highly corrosive substances. The samples should be processed as soon as they arrive in the laboratory.
Carefully cut the sample, weigh 10 g (with an error of at most ± 0.1 g) into an appropriate container, and mix with 90 ml of perchloric acid solution. Homogenize the mixture for 2 minutes in a mixer, then filter through filter paper.
The resulting extract can be stored for at least 7 days at a temperature between 2°C and 6°C.
Transfer 50 ml of the obtained extract into a vapor distillation apparatus.
To ensure that the extract has sufficient alkalinity, add a few drops of the silicone antifoam agent and 6.5 ml of caustic soda solution, then immediately start the distillation by vapor induction. Adjust the distillation rate so that 100 ml of distillate is obtained in 10 minutes. The distillate outlet tube is submerged in a container containing 100 ml of boric acid solution, to which 3-5 drops of the indicator solution are added. After 10 minutes, the distillation is completed. Raise the tube and wash it with water. Determine the content of volatile bases in the distillate solution by titration with the standard HCl solution. The final pH should be 5.0 (with an error of ± 0.1).
The analysis must be performed in duplicate. The analysis is considered correct if the difference between the two results does not exceed 2 mg/100 g. The titration is done with a standard HCl solution of 0.01 mol/l (0.01 N).
A control sample is also performed: instead of the extract, use 50.0 ml of perchloric acid solution. The content of easily hydrolyzable nitrogen (mg/100 g sample) is calculated using the following equation:

$$\text{Easily Hydrolyzable Nitrogen (mg/100g sample)} = (V1-V0) \times 0.14 \times 2 \times 100/M$$

where:
V_1 is volume of 0.01 M HCl solution (ml) used to titrate the sample;
V_0 – volume of 0.01 M HCl solution (ml) used to titrate the control sample;
M – mass of the sample (g).

Determination of sodium chloride (Mohr's Method)
Sodium chloride is added to food products to enhance flavor, increase preservation capacity, and, in meat products, as an agent to aid in the maturation (tenderizing) of meat during

production. In the aqueous extract obtained from the product under analysis, chloride ions are directly titrated with a silver nitrate solution in the presence of potassium chromate as an indicator. The chloride content is then calculated and expressed as sodium chloride equivalent.
Reagents: Silver nitrate 0.1 N; Potassium chromate, saturated solution (indicator).
Prepare the aqueous extract as per previous instructions. For dry products, the extraction time is longer (about 1 hour). To aid extraction, the flasks may be kept for about 30 minutes in a water bath at moderate temperature (55-60°C).
From the filtered aqueous extract, measure 10 ml into a 100 ml Erlenmeyer flask, add a few drops of potassium chromate, and titrate with 0.1 N silver nitrate solution while continuously stirring. The endpoint of the titration is considered when the color changes abruptly from light yellow to persistent orange. From this point onward, a drop of excess silver nitrate causes the color to shift to brick-red.
The total chloride content, expressed as sodium chloride %, is calculated using the following formula:

$$NaCl\ \% = (0.00585 \times V \times 10)/m \times 100$$

where:
V is volume of 0.1N silver nitrate solution (ml);
0.00585 – amount of sodium chloride (g) corresponding to 1 ml of 0.1 N silver nitrate;
10 – the ratio between the total volume of the aqueous extract (100 ml) and the volume of extract taken for analysis (10 ml);
M – mass of the sample (g) taken for analysis.
For easier calculation, instead of using 0.1 N silver nitrate solution, you can use a 2.906% silver nitrate solution. In this case, 1 ml of the silver nitrate solution corresponds to 0.1 g of sodium chloride, provided the procedure is followed exactly as described.

Determination of nitrites content (Griess Method)
Sodium or potassium nitrites are commonly used in meat product technology due to their ability to combine with the natural pigment of meat (myoglobin) to form a red complex that stabilizes with heat. Nitrites also combine with the natural pigment of blood (hemoglobin) to form a similar complex. Along with other curing agents (sodium chloride, nitrates, phosphates, ascorbates), nitrites play a positive role in improving the preservation capacity of meat products by inhibiting the growth of spoilage bacteria. However, free nitrites are considered potentially carcinogenic because they can combine with certain amines formed during the meat maturation process or during gastrointestinal digestion, forming nitrosamines, substances known for their carcinogenic effect. Nitrites can combine in an acidic medium with a primary aromatic amine to form a diazonium salt. If this salt is condensed or coupled with another primary aromatic amine, a colored complex is formed, which follows Beer's law.
Apparatus and reagents: Photocolorimeter or spectrophotometer; Acetilic solution of alpha-naphthylamine; Acetilic solution of sulfanilic acid; Saturated aqueous solution of mercuric chloride; Standard scale for comparison.
Take 12 uniformly calibrated, clean, and colorless test tubes and number them from 1 to 12. In each test tube, introduce an amount of $NaNO_2$ standard solution equivalent to the test tube's number (1 ml up to 12 ml). Then add 1 ml of the Griess complex reagent, followed by 0.5 ml of Griess A reagent, and then 0.5 ml of Griess B reagent. Complete the test tube to a final total volume of 13 ml.
Take 10 g of the sample to be analyzed, finely chop it, and place it into a Berzelius beaker. Add 100 ml of distilled water and leave it at room temperature for 30 minutes. Then filter it. In a test tube, take 1 ml of the filtered solution, 1 ml of Griess reagent, and 11 ml of distilled water. Homogenize the contents of the test tube and after 20 minutes, compare the obtained color with the standard scale.
The nitrite content of the sample, expressed as mg%, is equal to the number of the test tube on the standard scale corresponding to the observed color. If the color of the solution in the examined test tube is more intense than the color of the last test tube on the scale, dilute the extract 1/1 and reassess. The nitrite content will be the scale value multiplied by two.
In case of dispute, nitrite determination is done using the modified Griess method, which uses a spectrophotometer (Spekol) to read the color extinction.

Kreis reaction

The Kreis reaction qualitatively determines the oxidation stage of fat in meat and meat products, as well as in animal fats. The method's principle is based on the extraction of fat from the product and its treatment with fluoroglucine in the presence of hydrochloric acid. The oxidation degree is assessed by the intensity of the color obtained.

Reagents: Fluoroglucine; Hydrochloric acid; Ethyl ether. Place 10 g of fat into a test tube and heat at 105°C for 10-20 minutes until it melts. Mix 1 ml of the melted fat with 1 ml of HCl and 1 ml of fluoroglucine. Homogenize and observe the color of the liquid. Negative reaction: when the liquid remains colorless; Slightly positive reaction: appearance of a pink color of varying intensities; Positive reaction: appearance of a red color with a purplish hue (Banu, 2009).

RESULTS AND DISCUSSIONS

The analysis revealed significant physico-chemical differences between Bănățean Salami and Chicken Breast Frankfurters, influenced by their processing methods. Bănățean Salami had a moisture content of 40-45%, significantly lower than Chicken Breast Frankfurters, which exhibited a moisture percentage of 55-60%. The lower moisture content in Bănățean Salami is a result of fermentation and drying, contributing to its firm texture and extended shelf life.

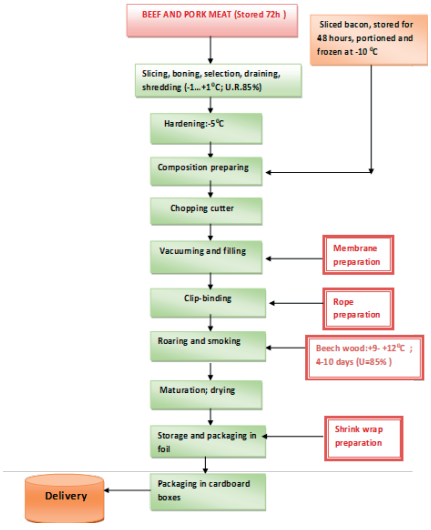


Figure 1. Technological flux for Bănățean Salami

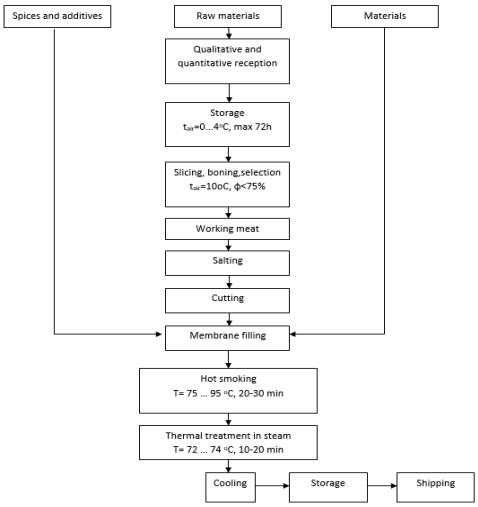


Figure 2. Technological flux for Chicken Breast Frankfurters

Conversely, the higher moisture content in Frankfurters enhances its softness and ease of consumption. The increased water retention in frankfurters contributes to their juiciness, an essential quality aspect preferred by many consumers, particularly for quick meals and fast-food applications. Moisture levels also influence microbiological stability, with drier products like salami being less prone to spoilage.

The fat content varied significantly, with Bănățean Salami containing 25-30%, whereas Chicken Breast Frankfurters had a much lower fat percentage (8-12%). This difference affects both texture and flavor, as the higher fat content in Bănățean Salami contributes to its rich mouthfeel and intense taste. The protein content was relatively high in both products, with Bănățean Salami containing 22-24% and Chicken Breast Frankfurters 18-20%, confirming their nutritional value. The higher fat content in salami also contributes to its distinct marbling, which plays a role in sensory perception, particularly in terms of tenderness and juiciness.For the products Salam Bănățean and Chicken Breast Sausages, the physico-chemical analyses of technological interest that were determined include: moisture, salt content, protein content, total fat, easily hydrolyzable nitrogen, and nitrite content. Based on the analysis of these characteristics, it can be stated that, from an organoleptic standpoint, all the

types of salami included in the study comply with the admissibility limits. Therefore, the products are manufactured according to the recipe and working instructions, and the raw materials, auxiliary ingredients, additives, and casings meet the requirements set by Regulation No. 825/2004/EC, Regulation No. 853/2004/EC, Directive 2002/99/EC, and Order 560/08.01.2007.

Table 1. Organoleptic characteristics of Bănățean Salami and Chicken Breast Frankfurters

Sortiment	Characteristics			
	Shape and size	**Aspect**	**Taste and smell**	**Texture**
Bănățean Salami	***Cylindrical sticks, 50-60 cm long and 40-70 mm in diameter***	Dry surface with small wrinkles, red-brown color, with rare (permitted) mold or good-quality yeast spots, composition with pieces of meat, fat, and rare mustard seeds	***Pleasant taste and smell of raw matured product, spices, and additives***	***Elastic to very firm***
Chicken Breast Frankfurters	Cylindrical pieces, 22-24 mm in diameter and 140-160 mm in length, twisted in strings	Clean surface, non-sticky, without water or fat accumulation at the ends or under the casing, continuous and undamaged casing, pale pink color	Pleasant, slightly salty, characteristic of the spices used, without foreign tastes or smells	Elastic

Table 2. Salt and water content of the meats analysed

PROBES	**Chemical parameter**			
	Experimentally determined values		***Permitted values***	
	Apa (%)	**NaCl (%)**	**Apa (%)**	**NaCl(%)**
Bănățean Salami	**25,5±0,2**	**3,30±0,1**	**Max.30**	**Max. 3,6-5,5**
Chicken breast Frankfurters	**62,70±0,1**	**2,10±0,15**	**Max.70**	**Max. 3**

Based on the analysis of these values, it is observed that the highest water content is found in the Chicken Sausages, while the lowest is in the Bănăţean Salami, and this correlates positively with the manufacturing technology (Bănăţean Salami is a dry-cured product). From a physico-chemical standpoint, both types of products analyzed met the admissibility conditions specified by the applicable Orders and Regulations. The same conclusions are drawn from the graphical analysis of the moisture and sodium chloride content in the analyzed product types.

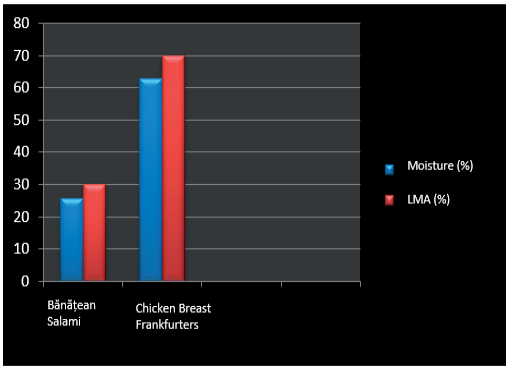


Figure 3. Variation of moisture in the analysed products

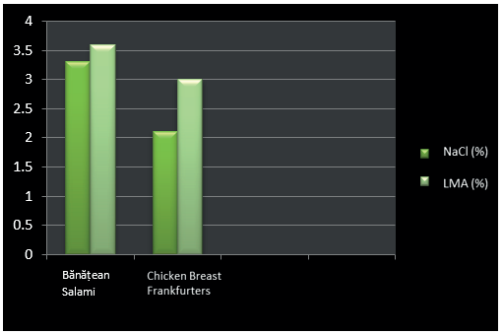


Figure 4. Variation of salt in the analysed products

It is well known that the nutritional value of cooked and smoked meat products is primarily determined by their protein and fat content.

Table 3. Protein and fat contents of the analysed products

PROBES	**Chemical parameter**			
	Experimentally determined values		***Permitted values***	
	Total Proteins (%)	**Fat (%)**	**Total proteins (%)**	**Fat (%)**
Bănățean Salami	**29,1±0,10**	**32,3±0,10**	**Min.11**	**Max.40**
Chicken Breast Frankfurters	**12,70 ±0,10**	**24,3 ±0,10**	**Min. 10**	**Max. 26**

The analysis of these values shows that Banat Salami has the highest protein content (29.1% compared to 12.7% for the Sausages), with both

products falling within the minimum admissibility limits (min. 11% for Salami and min. 10% for Sausages). This confirms that Banat Salami contains high-quality meat with a high biological value.
Regarding fat content, Banat Salami is also superior to the Sausages. Moreover, the manufacturing technology for Banat Salami involves the inclusion of beef and pork (in a 1:2 ratio) and the addition of fat.
The same conclusions can be drawn from the graphical analysis of the fat and protein content in the analyzed meat product samples (Figure 5).

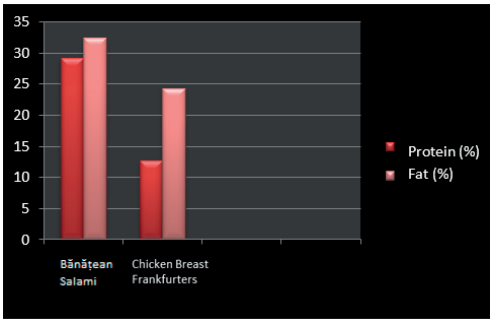


Figure 5. Variation of protein and fat content in the analysed products

In meat products (ham, salami, sausages), nitrites and nitrates are commonly used to maintain a pinkish-red color and for their bacteriostatic effects and flavor development in theproducts.To ensure even distribution throughout the meat mass, nitrates and nitrites are added to salt or brine. Under the action of reducing microorganisms in the brine and meat, nitrates are converted into nitrites. These nitrites oxidize myoglobin and hemoglobin in the blood into nitrosomyoglobin and nitrosohemoglobin, which maintain their red color during the meat treatment process. Without them, boiled or scalded salamis would turn a grayish color.In Romania, the concentration of nitrites in finished products is limited to a maximum of 70 mg/kg, while in some countries, it can be as high as 200 mg/kg.Based on these considerations, we aimed to determine the nitrite content in the two analyzed varieties, compared to their freshness, assessed by the Kreis reaction (Table 4).
From the perspective of nitrite content, it falls within the admissibility limits for all analyzed varieties, and according to the Kreis reaction, all productswere fresh.For the products included in the study, samples were sent to the laboratory for microbiological examination.

Table 4. Nitrites contents and Kreis reaction

PROBES	Chemical parameter		
	Experimentally determined values		*Permitted values*
	Nitrites (mg/100g)	Kreis Reaction	Nitrites (mg/100g)
Bănățean Salami	2,50±0,2	Negative	Max.7
Chicken Breast Salami	4,10 ±0,10	Negative	Max.7

The microbiological examination of the studied products included the following determinations: *Escherichia coli*, *Salmonella*, and *Listeria*, according to Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs, amended by Commission Regulation (EC) No. 1441/2007 of December 5, 2007, and the self-control program developed based on this Regulation.
The following indicators were determined: the number of *E. coli* bacteria, the presence of *Salmonella* germs, and the presence of *Listeria* germs, using the methods provided by the official standards in force.For determining the *Salmonella* bacteria in the analyzed products, 5 samples were collected and analyzed (one sample for each product), totaling 20 samples (5 x 0.5 kg for each sample) during the above-mentioned period. The same number of samples was collected for the determination of *Listeria monocytogenes* and *Escherichia coli*. All the results obtained were summarized in Table 5 (Banu C., 2009).

Table 5. Microbiological analysis of the chosen products

ANALYSED PRODUCT	VALUES		
	E. coli	*Listeria*	*Salmonella*
BĂNĂȚEAN SALAMI	<10	Absent	Absent
CHICKEN BREAST FRANKFURTERS	<10	Absent	Absent

Of all the samples analyzed, 100% showed results that were in compliance with Regulation (EC) No. 2073/2005 on microbiological criteria

for foodstuffs, as amended by Regulation (EC) No. 1441/2007.

CONCLUSIONS

This study successfully evaluated the technological, physicochemical, microbiological, and sensory characteristics of Bănățean Salami and Chicken Breast Frankfurters. The findings confirmed that both products adhere to industry quality standards and consumer expectations. Understanding the differences between traditionally fermented meat products and emulsified, thermally processed alternatives can help both manufacturers and consumers make informed choices. Future research should explore alternative preservation techniques and ingredient modifications to further enhance the nutritional profile and safety of processed meat products, particularly in response to changing dietary trends and consumer health concerns.

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STUDY ON THE SHELF LIFE OF VACUUM-PACKED MATURED BEEF

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Abstract

The research conducted on the quality and safety characteristics of matured beef, packaged in polyethylene foil, aimed to optimize the shelf life of this food product. The investigations were carried out on samples taken from the local producer and the organoleptic properties (external appearance, cross-sectional appearance, consistency, odor and color) which present appreciable characteristics, and the physicochemical indices (pH value, protein, fat content, peroxidase and peroxidase reaction, etc. were evaluated) demonstrated the requirements in parametric terms. Following microbiological investigations, no pathogenic microorganisms were detected such as: Salmonella spp., E. coli beta-glucuronidase positive, UFC/g, Listeria monocytogenes, etc., which would affect the health of consumers. Heavy metal residues (Cd, Pb) do not exceed the maximum permitted limits for this product, which proves the conformity and safety of matured beef during the shelf life.

Key words*: heavy metal residues, mature beef, shelf life.*

INTRODUCTION

Whether animal, plant or mixed, food is essential for humans. This is because it provides energy and the nutrients needed to maintain health. The human body remains healthier when the diet is balanced, diversified and correctly composed in terms of calories and respecting the optimal ratio of main substances (carbohydrates, proteins, lipids, dietary fibers), (Hodoşan et al., 2023). Food is the most important element, which affects the body, and the maintenance of human health is closely linked to the "health of food".

The main purpose of food safety is to ensure the protection of human health at a high level, and the main objective is to represent the interests of consumers with regard to food products. The EU food safety policy is based on the "farm to fork" concept, which is a holistic approach that vertically integrates every stage of the product approval process, from feed production, plant health, animal welfare, primary production and secondary processing, packaging, storage, retail, delivery for import and export (Georgescu et al., 2000; Diaconescu et al., 2007).

Meat is consumed worldwide due to its high nutrient content, aroma and taste appreciated by consumers. Various health deficiencies (protein malnutrition, iron deficiency, anemia, vitamin deficiencies) can be prevented or corrected by consuming meat and other animal products (Ianiţchi et al., 2024). Due to these special properties, meat consumption has increased significantly, especially in developing countries, and is expected to double by 2050.

Beef, in particular, is appreciated for ensuring a healthy and balanced diet, due to its important content of macro and micro nutrients, proteins, proteolytic enzymes, etc. However, beef requires a longer time for culinary preparation, compared to other types of meat, meanwhile prolonged heat treatment can have negative effects on the chemical composition of the product.

According to studies on meat consumption - the quality of red meat consumption depends on aging (maturation) and this has been included in various guidelines for food quality (Tudor et al., 2009; Pop et al., 2009).

To improve the properties of meat, especially taste and aroma, juiciness and tenderness, meat can be subjected to the maturation process (Oprică, 2011; EFSA, 2022a; Jeremiah, 2001; Air Products SAS, 2016). Another form of improving the tenderness and juiciness of meat is tumbling or massaging, which also ensures a

homogeneous dispersion of salting and flavoring components (Pătrașcu et al., 2011).

Matured beef is a product appreciated by consumers in several countries, in recent years it has also been of interest to the Republic of Moldova.

The main benefits of meat maturation are:

• reducing meat hardness, improving consistency;
• increasing protein solubility, hydration level;
• increasing protein digestibility due to the destruction of the actomyosin complex;
• improving the process of collagen digestibility;
• forming the taste and aroma of meat due to enzymatic transformations of proteins and other enzymes;
• dry maturation has a positive effect in reducing the microbiological load on meat over longer periods, due to the combined effect of cooling and drying.

During maturation, the formation and accumulation of products of enzymatic decomposition of proteins and peptides (glutamic acid, sulfur-containing amino acids), nucleotides (inosine, hypoxanthine, etc.), carbohydrates (glucose, fructose, pyruvic and lactic acids), lipids (low molecular weight fatty acids), as well as creatine, creatinine and other nitrogenous extractive substances, precursors of the taste and aroma of meat, occurs.

Due to the formation of a mold crust on the surface of the meat, during the maturation process, the meat is protected from the effects of pathogenic microflora, softens and acquires a pleasant sweetish (corn) taste. Before sale, cooking, the crust is, of course, removed.

Thus, in the meat maturation process, a significant improvement in organoleptic and technological characteristics, nutritional value compared to refrigerated, fresh meat is observed (UKHSA, 2024; Farber et al., 2006; Jeremiah, 2001; EFSA, 2022b).

MATERIALS AND METHODS

Laboratory investigations were carried out to determine the quality (physico-chemical), safety, microbiological and sensory evaluation indices during the storage of 21 days matured beef (at temperature 0-2°C) in order to establish the required shelf life, packaged in polyethylene foil, in pieces with a net mass of 0.2-0.4 kg.

The analyses were carried out during September-October 2023. The evaluation of the quality and safety parameters of matured meat, packaged in polyethylene foil, produced for public consumption was carried out by sampling three batches of meat with different production dates. The meat was subjected to the dry maturation process, half-carcasses, quarters and pieces on the bone in unpackaged form, in refrigerated warehouses, on shelves (with controlled temperature and humidity) at a temperature of 0-2°C and a relative air humidity of 75-80% for 21 days, with the provision of a constant air flow with an indicator of 0.5 to 2 m/s. At the end of the maturation period (after 21 days), the cut meat, sliced into smaller pieces (0.2-0.4 kg), is vacuum-packed for transmission to the retail chain, where it continues to be stored under similar conditions at a temperature of 0-2°C and a relative air humidity of 75-80%.

Laboratory investigations were carried out in the Republican Center for Veterinary Diagnostics, in the Food Products Testing Laboratory. Laboratory evaluations included organoleptic, physicochemical, microbiological and safety indices, which were compared with the requirements of the standards in force for this category of products.

The frequency of research for "Mature beef" prepared according to the manufacturer's technological instructions was established as follows: initially (background), after 5 days, 10 and 13 days of storage. Investigations for each stage and date of production were carried out on intact, previously unopened samples.

All investigations were carried out in an accredited laboratory using classical reference methods. Heavy metals were determined by atomic absorption spectrometry with a graphite furnace using the standardized method EN 14083 and EN 13806.

The determination of quality indices was made using classical physicochemical methods: protein determination using the ISO 937 method, fat content using the standardized method ISO 1443.

The microbiological indicators were determined using classical reference methods according to EN and ISO standards: Colony count at 30°C, ufc/g – ISO 4833; *E. coli* beta-glucuronidase positive, ufc/cm^2 – ISO 16649-2; *Enterobacteriacae*, log ufc/cm^2 – ISO 21528-2;

Salmonella spp. – ISO 6549; *Listeria monocytogenes* in 25.0 g – ISO 11290.
The obtained results were compared with the requirements of the regulations in force for this product category; Government Decision no. 696/2010 for the approval of the Requirements regarding the production, import and placing on the market of raw meat; Government Decision no. 221/2009 – Rules regarding microbiological criteria for food products; Government Decision no. 520/2010 – Sanitary Regulation regarding contaminants in food products.

RESULTS AND DISCUSSIONS

The evaluation of the quality and safety of mature beef for 21 physical days packaged in polyethylene foil in order to ensure the stability of the shelf life requires the study of quality indices, namely: safety and microbiological.
Following the laboratory tests carried out, it was established that matured beef for 21 days met the requirements of the normative documents in force, Government Decision No. 696/2010 - Requirements for the production, import and introduction on the market of meat - raw material, and from a nutritional point of view is a rich source of protein (21-22%), and moderate source of fat (4.7%).
Laboratory investigations for the assessment of organoleptic indices, presented that pieces of beef have appearance of marbled meat (with the presence of strips/threads of fat between the muscle fibers). The surface of the meat is clean, without foreign impurities and visible blood stains, with a glossy surface, of a bright, natural color from red to burgundy, without stains, the threads of fat are white. In the package without leaked liquid: in the section - the samples of matured beef for 21 days presented well-developed muscles, the meat slightly moist, shiny, with thin strips of intermuscular fat. The meat is red-burgundy in color, uniform over the entire surface of the muscle, without gray spots. The threads of fat are white. The elastic consistency of the meat, firm, the dimple from pressing with a finger quickly disappears. When opening the package with meat and cutting the meat in section, a pleasant smell was felt, characteristic of fresh meat, without foreign odor.
Thus, beef matured for 21 days, packaged in polyethylene foil and stored at a temperature 0-4°C retained its sensory characteristics (appearance, appearance in section, consistency, odor) for 13 days, without changing the organoleptic characteristics and correspond to the normative documents GD no. 696/2010 and the Technological Instruction.
Along with the organoleptic indices, the physicochemical quality indices of beef matured for 21 days, packaged in polyethylene foil were also assessed (Table 1).

Table 1. Physico-chemical indices of matured beef

Indices examined	Regulatory requirements	Results achieved			DN to test method
		Batch I (n=3) X ± Sx	Batch II (n=3) X ± Sx	Batch III (n=3) X ± Sx	
Protein mass fraction, %, min.	De facto	22.295 ± 0.465	21.288±0.071	21.125±0.275	ISO 937
Fat mass fraction, %, max.	De facto	2.300±0.050	4.750±0.076	4.775±0.247	ISO 1443
pH value, un.	De facto	5.750±0.050	5.780±0.082	5.775±0.076	ISO 2917

Following the analysis of the meat, proteins values of 21.120- 22.29% were recorded, which are specific and ISO 937 compliant.
The protein value of the meat is also influenced by the way it is prepared (the highest value is recorded in boiling or frying, when the internal temperature of the meat does not exceed 65-70^{0}C). The fat content depends largely on the degree of fat cover and the amount of adipose tissue. The fat content in beef varies (1-3.5%), which is why beef is considered dietetic.
After the research carried out on the matured beef samples, it can be said that the batches of meat examined had fat content values ranging from 2.1% to 5.1%. The results obtained meet the requirements in force and comply with ISO1443.
It should be noted that meat quality depends on the initial microbiological contamination as well as the pH value (when the meat has a pH above 6.2, its shelf life is reduced).

Acidity is one of the main factors that inhibit the development of bacterial microflora, thereby preventing spoilage.

The concentration of hydrogen ions in meat depends on the glycogen content in the muscles at the time of slaughter. In living muscle, the pH ranges between 7.0 and 7.5, and after the animals are slaughtered, under normal conditions, the pH value of the meat decreases relatively quickly due to the process of glycolysis, which leads to the formation of lactic acid. As a result, 12 to 14 hours after slaughter, the pH reaches its final value of 5.4-5.6. After that, the pH value stabilizes.

Therefore, pH values between 5.6 and 6.0 indicate an environment in which microbial growth is reduced or slowed down, and this favors the stability of the sensory attributes of the meat. At the same time, the variation in pH influences the technological properties of meat (water retention capacity, hydration capacity), which improve with increasing pH (Ianiţchi et al., 2019).

In order to monitor possible lipid oxidation processes, which may occur as a result of biochemical processes, tests were carried out to determine the peroxide value and the acidity value in the fat extracted from beef matured for 21 days. Thus, in Figure 1, the results are shown regarding the acidity values in beef matured for 21 days and stored at t =0-4°C.

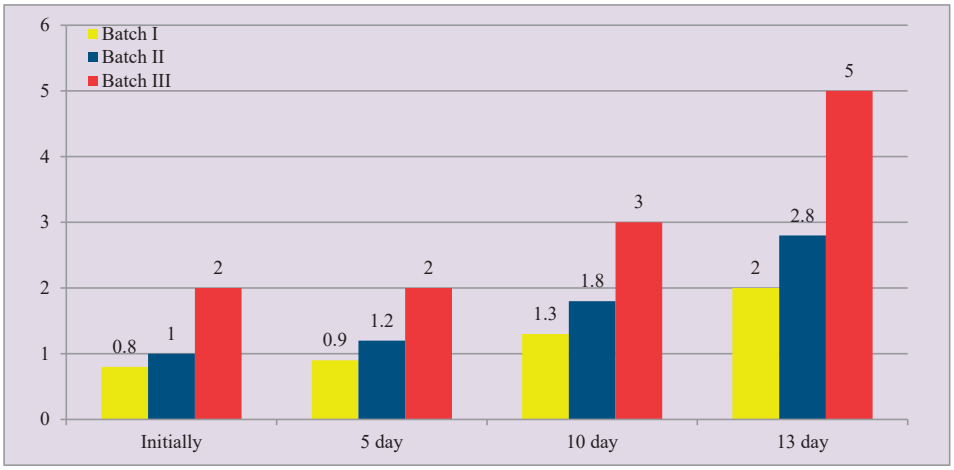


Figure 1. Variation in acidity index of matured beef

In accordance with the obtained results, the acidity index evaluated in the 21 day matured beef samples taken into study for all three batches showed values ranging from 0.8 to 5.0 on the 13th day. These values show that the acidity index on the 10th day was 3 mg KOH/g, which confirms that the beef is considered fresh, because, according to GOST 33818, beef is considered fresh if the acidity is up to 4 mg KOH/g. Next, in Figure 2, the results of laboratory tests on peroxide values in matured beef for 21 days and stored at temperature 0-4°C are presented.

Therefore, laboratory investigations carried out on possible oxidation processes (hydrolytic rancidity, aldehyde rancidity) on the 21 day matured beef samples (Figure 2) demonstrated that samples corresponds in terms of quality, because they were not affected the aroma, texture, color, nutritional value, and neither acidity index and peroxide, according to the GOST 34118 standards.

An indication of meat quality preservation is the peroxidase reaction. The essence of the reaction is that the peroxidase decomposes hydrogen peroxide, and the resulting atomic oxygen rapidly oxidizes benzidine to paraquinodiimide, which with benzidine residues forms a blue-green compound that turns brown in a few seconds, a characteristic reaction for fresh meat. When the initial compound turns blue-green and very slowly turns brown, the reaction is partially negative and the meat is suspect regarding freshness. In case of negative reaction, the

compound immediately turns brown, a process characteristic of spoiled meat. For beef matured for 21 days, peroxidase tests were performed for all three batches of products at each stage: initially, after 5, 10 and 13 days of storage (Table 2).

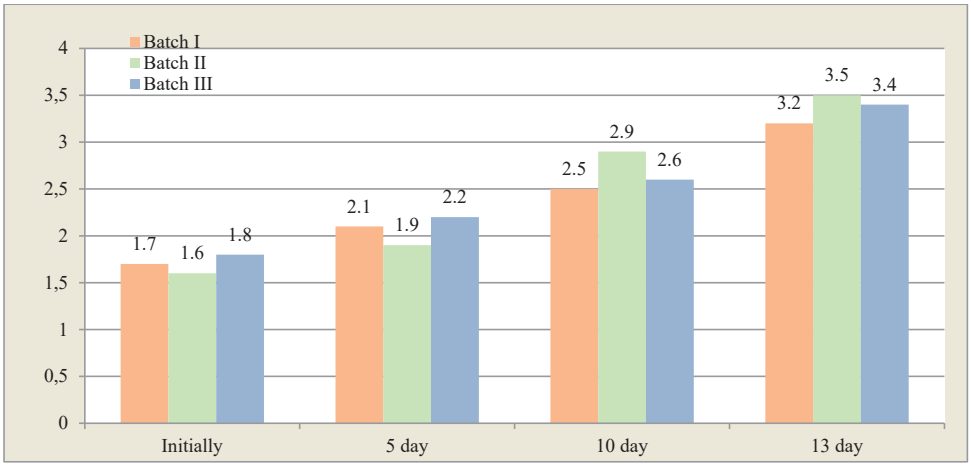


Figure 2. Variation of peroxide value in matured beef

Table 2. Evaluation of peroxidase and Kreiss reaction in matured beef

Indices	Regulatory requirements	Results achieved			DN to test method
		Batch I (n=3) X ± Sx	Batch II (n=3) X ± Sx	Batch III (n=3) X ± Sx	
Peroxidase reaction	Positive reaction	Positive reaction	Positive reaction	Positive reaction	GOST 7702.1
Kreiss reaction (H_2S)	Negative reaction	Negative reaction	Negative reaction	Negative reaction	SR 9065-11

However, the peroxidase activity depends on the pH value:
- up to pH=6.0 the peroxidase reaction is positive;
- at pH=6.1-6.2 the peroxidase reaction is suspect;
- over pH 6.2 the peroxidase reaction is always negative.

Thus, at all stages of research the peroxidase reaction for the samples taken into study was positive – a characteristic reaction for fresh meat, or the pH for meat up to 10 and 13 days of storage was maintained within the limits of 5.5-5.6, a specific environment for the positive peroxidase reaction. Determining the stage of fat oxidation by the Kreis Reaction helps to identify the presence of epihydric aldehyde, which appears as a result, of the oxidative processes of linoleic acid in an advanced stage of fat oxidation. Also, a positive Kreis Reaction confirms an advanced stage of alteration in which significant organoleptic changes in taste, color and smell occur.

According to the obtained results, beef matured for 21 days corresponds in terms of quality, because the Kreis Reaction determined in the samples taken in the study was negative.

According to the initial research program, samples of matured beef were subjected to investigations to determine the safety indices: heavy metals (Pb, Cd) (Table 3).

Table 3. Safety indices for matured beef

Indices	Normative Requirements	Results obtained			DN to test method
		Batch I (n=3) X ± Sx	Batch II (n=3) X ± Sx	Batch III (n=3) X ± Sx	
Pb, mg/kg	max. 0.1	0.020±0.003	0.060 ± 0.005	0.050 ±0.003	SM SR EN 14082:2006
Cd, mg/kg	max. 0.05	<0.005	<0.005	<0.005	SM SR EN 14082:2006

The results presented in Table 3, referring to the safety of the 21 day matured beef, demonstrate that all the samples investigated, both for the content of Pb (lead), mg/kg, and for Cd (cadmium), mg/kg, presented values that do not exceed the maximum permissible limits, which confirms that the matured beef is safe and meets the requirements of the normative acts in force - Government Decree no. 520/2010 - Sanitary Regulation on contaminants in food products.

The intensity of microbiological processes depends on the composition and properties of the meat: the type of raw material, its water content, pH value, water activity, surface condition (presence of cuts, drying of the crust, etc.). The use of protein films with a protective role can be a solution for limiting microbiological contamination of meat (Cercel et al., 2017). The most important factor affecting the shelf life of raw meat is the initial microbial contamination. Contamination of meat carcasses and other slaughter products occurs both endogenously and exogenously. During the storage period, microbiological indices were examined, which could modify the microflora and product quality stroge – colony count at 30^0C, *E. coli* beta-glucuronidase positive, *Enterobacteriaceae*, *Salmonella* spp. in 25.0 g, and *Listeria monoytogenes* in 25.0 g, and the results obtained on microbiological testing are presented in Table 4.

Table 4. Dynamics of microbiological indices during shelf-life

Sample Name/ Defrosting Date	Shelf Life, Days	Colony Count at 30°ufc/g	*E. coli* Beta-Glucuronidase Positive, ufc/cm^2	*Enterobacteriaceae*, log ufc/cm^2	*Salmonella* spp (detection) in 25.0 g	*Listeria monocytogenes* in 25.0 g
Test Method		SM EN ISO 4833-1:2014	SM SR ISO 16649-2:2011	SM ISO 4831:2010	SM EN ISO 6549-1:2017	SM EN ISO 11290-1:2017
Normative Requirements		max. $1*10^6$	max. 500	Max 2,5	Not Allowed	Not Allowed
Matured Beef for 21 Days - Lot I t = 2±0.6 °C (storage temperature) Packaged in PE film m.n= 0.2-0.4 kg	initially	$4.3*10^4$	<10	<1	Not Detected	Not detected
	5	$2.7*10^5$	<10	2.0	-	Not detected
	10	$1.3*10^5$	<10	<1	-	Not detected
	13	7.4*105	<10	<1	-	Not detected
Matured Beef for 21 Days - Lot II t = 2±0.6 °C (storage temperature) Packaged in PE film m.n= 0.2-0.4 kg	initially	$3.5*10^3$	<10	1.7	Not Detected	Not detected
	5	$1.5*10^3$	<10	<1	-	Not detected
	10	1.7*105	<10	<1	-	Not detected
	13	$1.9*10^5$	<10	<1	-	Not detected
Matured Beef for 21 Days -Lot III t = 2±0.6 °C (storage temperature) Packaged in PE film m.n= 0.2-0.4 kg	initially	$2.1*10^4$	<10	<1	Not Detected	Not detected
	5	$2.5*10^5$	<10	<1	-	Not detected
	10	$2.2*10^5$	40	1.5	-	Not detected
	13	$6.5*10^5$	50	1.6	-	Not detected

The results presented regarding the microbiological indices did not show deviations from the admissible limits for the samples taken into study, and, the number of colonies at 30^0C changed insignificantly, with a slight increase, for all three batches, but which fell within the admissible limits of the normative acts in force. However, the matured beef for 21 days is safe from a microbiological point of view even after 13 days of storage at t=0-4^0C.

CONCLUSIONS

The organoleptic characteristics assessed in matured beef packaged in polyethylene foil and stored at a temperature of 0-4^{0}C preserved its maintained (appearance, cross-sectional appearance, smell, consistency) for 13 days without changing them. These are in accordance with Government Decree no. 696/ 2010.

The evaluated physicochemical indices (fat content, protein content and pH value) in matured beef presented concrete, compliant data that fall within the requirements of the regulations in force. Thus, the fat content in batch I is 2.300%, batch II is 4.750% and batch III is 4.775%. The protein content in batch I is 22.295%, batch II - 21.288%, batch III - 21.125%. The pH value of batch I is 5.750, batch II - 5.780%, batch III - 5.775%, results that fall within the limits of the requirements of the regulations in force for this product category.

The results on the safety indices (Pb and Cd), correspond to the requirements of the regulations in force, respectively Government Decision no. 520/2010.

Following the evaluation of the microbiological indices, it was established that the samples are safe and compliant even after 13 days of storage at t=0-4°C, with Government Decision 221/2009

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A CRITICAL REVIEW ON THE INFLUENCE OF BIOACTIVE COMPOUNDS ON MEAT AND THEIR EFFECT ON *Salmonella* CONTROL: PROMOTING FOOD SAFETY AND QUALITY

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Abstract

Meat, an essential component of the human diet, is susceptible to contamination by pathogens such as Salmonella, posing significant public health risks. In this context, bioactive compounds offer innovative solutions for enhancing food safety through their antimicrobial and antioxidant effects. This study explores the influence of bioactive compounds on meat, with a focus on preventing and controlling Salmonella contamination. Studies show that their application in surface treatments or active packaging materials inhibits the development of pathogenic bacteria, including Salmonella, thereby reducing contamination risks. In addition, plant extracts rich in polyphenols and flavonoids contribute to meat stabilization by preventing lipid and protein oxidation. Modern processing methods allow the incorporation of these bioactive substances into meat products through techniques such as marination, injection, or active packaging. This study highlights the importance of technological advancements and interdisciplinary collaboration for harnessing the potential of bioactive compounds for combating Salmonella and ensuring safe and high-quality food.

Key words*: bioactive compounds, contamination, essential oils, meat, Salmonella.*

INTRODUCTION

Food safety is a global priority with a direct impact on public health and the economy. The meat industry is one of the sectors most exposed to microbiological contamination, and issues related to meat safety can have serious consequences for consumer health, often leading to the recall of contaminated products. The greatest risks are associated with the presence of microbial pathogens, particularly bacteria. In recent years, several outbreaks of foodborne illnesses caused by pathogenic bacteria have drawn attention to meat safety and have become a major societal concern (Sofos, 2007).

Among the pathogens responsible for foodborne illnesses, *Salmonella* is one of the most important. As reported by the European Food Safety Authority (EFSA), this microorganism is a leading cause of salmonellosis, with more than 91,000 cases reported annually in Europe. *Salmonella* is commonly found in eggs and raw meat from pigs, turkeys, and chickens, and is transmitted to humans through contaminated food. The World Health Organization (WHO) confirms that *Salmonella* infections are among the most common causes of foodborne illness worldwide (WHO, 2022). Meat and meat products are important sources of high-quality protein, essential amino acids, B vitamins, and minerals. However, the chemical composition of meat makes it a perishable product, vulnerable to spoilage at all stages of processing, distribution, and storage. Microbiological contamination can be caused by both spoilage bacteria, such as *Acinetobacter*, *Brochothrix thermosphacta*, *Enterobacter*, *Lactobacillus sakei*, and *Pseudomonas*, as well as yeasts and molds, which generate unpleasant odors and flavors (Jayasena & Jo., 2013).

Furthermore, certain pathogenic bacteria, including *Aeromonas hydrophila, Campylobacter jejuni, Clostridium perfringens, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica*, pose a major risk to public health. In addition, *Bacillus cereus* and *Clostridium botulinum* are bacteria capable of forming spores resistant to common heat

treatments. *Salmonella* contamination of food has become a global public health problem, a leading cause of foodborne diarrhea in both the EU and the USA, as well as the third leading cause of death associated with foodborne infections worldwide (Yang et al., 2015).

An important aspect of food safety is the implementation of effective strategies to prevent and control microbiological contamination. Traditional methods of pathogen reduction include heat treatments, the use of chemical preservatives, and controlled atmosphere packaging. However, these methods can affect the nutritional and sensory quality of meat products (Jayasena & Jo., 2013). For this reason, recent research has focused on the use of alternative solutions, such as the application of bioactive compounds with antimicrobial and antioxidant properties, offering a more sustainable approach to food safety.

MATERIALS AND METHODS

The data and information required to achieve the purpose of the paper were gathered through an assessment of the state of knowledge from the ScienceDirect, Google Scholar, PubMed, and Scopus databases. To gather relevant information, the following search terms were used: 'bioactive plant compounds', 'Salmonella contamination', 'meat preservation', 'essential oils', 'antimicrobial properties', 'Salmonella', 'meat', 'antimicrobial peptides', and 'food safety'. The included studies were published between 1999 and 2023, were available in English, and focused on evaluating the effects of bioactive compounds on *Salmonella* contamination in meat products. The selection was limited to articles published in peer-reviewed journals that described relevant experiments with bioactive compounds and provided clear evidence of their efficacy. Data from the included studies were analyzed narratively, focusing on the main findings related to the prevention of *Salmonella* contamination, identifying trends based on the types of compounds used and their impact on food safety.

RESULTS AND DISCUSSIONS

Studies on bioactive plant compounds have shown that they can play a key role in improving the safety and quality of meat, with significant antimicrobial effects, particularly against *Salmonella* contamination. Essential oils from plants such as oregano, thyme, and rosemary have proven effective in preventing Salmonella biofilm formation on meat surfaces. For instance, the essential oils derived from *Origanum vulgare* (oregano) and *Rosmarinus officinalis* (rosemary) were successful in eliminating biofilms of *Salmonella enteritidis* from stainless steel surfaces, with their effects varying over time and employing a multi-target approach on the bacterial membrane (Lira et al., 2020).

In addition, active compounds such as carvacrol and thymol, present in the essential oils of oregano and thyme, have demonstrated inhibitory effects on *Salmonella* biofilm formation at sublethal concentrations, although they failed to completely eliminate *Salmonella* biofilms (Soni et al., 2013). However, the use of high concentrations of essential oils may negatively affect the sensory characteristics of meat products, influencing their acceptability to consumers (Pietrasik et al., 2013).

Antimicrobial peptides, such as nisin Z, have also shown efficacy against *Salmonella* and play an important role in stimulating host immunity. Studies have suggested that their mode of action is similar to that of natural human defense peptides (Kindrachuk et al., 2013).

Despite promising results, using bioactive compounds in the meat industry faces challenges, such as low solubility, low bioavailability, and limited thermal stability.

The Role of Bioactive Compounds in Food Protection

Plants have long been a rich source of bioactive compounds, used not only as folk medicines (Chandrasekara & Shahidi, 2017) but also as natural preservatives for food since ancient times (Prakash et al., 2017). Currently, interest in these compounds has increased significantly due to food safety concerns and the desire to reduce the use of synthetic preservatives. Herbs are intensively studied in scientific research and are widely incorporated into the chemical and pharmaceutical industries owing to their antioxidant benefits (Nunes et al., 2016; Zugravu et al., 2022), antibacterial (Parham et

al., 2020; Trifunschi et al., 2022), and antifungal (Chojnacka et al., 2020; Di Sotto et al., 2020) properties.

In the food industry, the use of bioactive compounds as natural preservatives represents a promising alternative to traditional methods of preventing contamination. These compounds are effective in inhibiting the growth of pathogenic microorganisms and can also enhance the shelf life of food products, retaining their sensory characteristics (Ribeiro-Santos et al., 2017).

Plant-derived antimicrobials are the main category of natural preservatives, consisting of secondary metabolites that target microbial cells. Various parts of plants, including seeds, fruits, bark, leaves, and roots, contain these antimicrobials in abundance (McClements et al., 2021). Among the key bioactive compounds that play a protective role in food are phenolic compounds (such as simple phenols, phenolic acids, anthocyanins, flavonoids, and quinones), terpenes, catechins, alkaloids, saponins (such as dihydropyranones), and coumarins, which function as both antioxidants and antimicrobials (Bouvard et al., 2015; Velázquez et al., 2021).

There is considerable interest in essential oils, as they are natural substances and are classified as GRAS (Generally Recognized as Safe) food additives by the Food and Drug Administration (2016). In addition, they have received Qualified Presumption of Safety (QPS) status in both the USA and the EU (Saeed et al., 2019).

Due to their antimicrobial and antioxidant efficacy, polyphenols, terpenoids, alkaloids, and antimicrobial peptides are among the most studied classes of bioactive compounds. Polyphenols, found in plants, these substances are known for their effectiveness in reducing the growth of pathogenic bacteria, including *Salmonella* and *Listeria monocytogenes*, in meat products. Their functional mechanism involves disrupting the bacterial cell membrane and inhibiting the activity of enzymes essential for pathogen survival (Burt, 2004).

Antimicrobial peptides, such as nisin and pediocin, have widespread applications in the meat, dairy, and fermented beverage industries. They act by destabilizing the bacterial cell wall, leading to microbial cell death (Cotter et al., 2005). For example, nisin is approved as a food additive (E234) and is commonly used to extend the shelf life of cheeses and processed meats (Gharsallaoui et al., 2015).

An important challenge in the application of bioactive compounds is ensuring their efficacy in the food matrix. Factors such as solubility, bioavailability, and thermal stability can influence the antimicrobial effects of these substances. Therefore, current research is exploring innovative technologies, such as nanoemulsion encapsulation and antimicrobial active films, o optimize the stability and sustained release of bioactive compounds in food matrices (Ribeiro-Santos et al., 2017; Sharma et al., 2022).

The application of bioactive compounds in the meat industry is a viable strategy for enhancing food safety. The results demonstrate that these compounds have a significant antimicrobial effect on *Salmonella* spp., thereby helping to reduce the risk of contamination. Furthermore, certain bioactive compounds have been shown not only to inhibit bacterial growth but also to help maintain the sensory and nutritional characteristics of meat.

Bioactive Compounds and Their Effects on Meat

Bioactive compounds are natural or synthetic substances that, when taken in optimal doses, they contribute to human health by affecting specific biological pathway. These compounds can be found in various food sources (Yehmed et al., 2023) and feature a broad spectrum of biologically active compounds (Munekata et al., 2020).

Natural and Synthetic Sources of Bioactive Compounds

Within the food industry, bioactive compounds fall into two categories: natural and synthetic. Although synthetic variants are the most widely utilized and have regulated safe consumption limits, concerns over their possible harmful effects, including carcinogenicity (Georgantelis et al., 2007), have raised doubts about their safety. Consequently, the demand for natural alternatives has surged (Georgantelis et al., 2007). As a result, the search for natural compounds has increased (Bauer et al., 2001; Souza et al., 2006).

The natural sources of bioactive compounds in meat are diverse and include proteins, lipids, and minerals that are important for human health. Bioactive compounds are a heterogeneous group of substances with varying chemical characteristics, primarily found in plants and plant-derived matrices (Oswell et al., 2018). As widely reported in the scientific literature, antioxidants are utilized to prevent oxidation by interrupting the early stages or propagation of oxidation chain reactions (Gómez et al., 2019). Notably, several studies indicate that plant extracts can positively impact consumer health, may have a stronger antioxidant capacity than synthetic antioxidants (Zhang et al., 2018; Jayawardana et al., 2019). As a result, the activity of natural antioxidants offers protection to biologically significant cellular components against oxidative damage caused by reactive oxygen species (ROS) (Lin et al., 2020). Yet, in the meat industry, the utilization of bioactive compounds has been restricted by challenges such as poor bioavailability, rapid release, low solubility, and vulnerability to environmental stress. Process-related conditions, such as pH, temperature, oxygen and light exposure, and storage time, can also affect their efficacy (Aguiar et al., 2016).

Synthetic sources of bioactive compounds include food additives and supplements that can be used to improve the nutritional profile of meat. For example, butylated hydroxytoluene (BHT), propyl gallate (PG), butylated hydroxyanisole (BHA), and tert-butyl hydroquinone (TBHQ) are some of the most commonly used synthetic antioxidants (Takemoto et al., 2009). Scientific studies have revealed the toxicity of BHT, BHA, and TBHQ (Cruces-Blanco et al., 1999).

Based on scientific research, the acceptable daily intake of synthetic antioxidants has been adjusted. Given the potential risks associated with synthetic antioxidants, there has been a shift toward replacing them with natural antioxidants or blends of natural compounds, thereby lowering the consumption of synthetic antioxidants (Coneglian et al., 2011; Soares, 2002).

In addition to influencing meat quality, the use of bioactive compounds can play a important role in increasing the competitiveness of local producers and integrating them into the agri-food chain by promoting quality schemes and enhancing the added value of agricultural products. Thus, the development and certification of quality products contribute to the promotion of an organized food chain, support local heritage, stimulate rural tourism, and help develop competitive local brands. Studies conducted in Romania have highlighted an uneven distribution of certified products across regions, which indicates a significant potential for expanding certification and adapting the supply to meet consumer demands (Maloș & Maloș, 2016).

Influence of Bioactive Compounds on *Salmonella* Contamination

Salmonella infections are among the most common foodborne diseases worldwide. According to the WHO, *Salmonella* is responsible for millions of gastroenteritis cases annually, with many of these linked to eating contaminated meat (WHO, 2023). In the meat industry, sources of contamination include intensive handling, insufficient sanitation processes, and cross-contamination during meat processing (Fernandes et al., 2017).

From an economic perspective, *Salmonella* outbreaks result in significant losses (Kim et al., 2024). In particular, poultry and minced meat are considered the most vulnerable to contamination risks (Gonçalves-Tenório et al., 2018). Gram-negative bacteria tend to be less responsive to antimicrobial agents than Gram-positive bacteria. Several components of essential oils have been identified as effective antibacterial agents, including carvacrol, thymol, eugenol, perylaldehyde, cinnamaldehyde, and cinnamic acid, with minimum inhibitory concentrations (MICs) ranging from 0.05-5 $\mu l\ ml^{-1}$ *in vitro* (Burt, 2004). Herbs and spices, despite their potential as food preservatives, are still generally accepted as safe according to Burt (2004). The antimicrobial effects of essential oils have been investigated in fresh meat, although limited studies focus on their application in dried meat products (Martín-Sánchez et al., 2011). Different essential oils exhibit varying inhibitory effects (García et al., 2011), so *in vitro* testing is essential before their use in food (Gutierrez et al., 2009). Sensory properties are

important for consumer acceptance, and the organoleptic effects of essential oils must be carefully managed, as high concentrations needed for antimicrobial efficacy may result in the rejection of the product due to unpleasant odors and tastes (Pietrasik et al., 2013).

Terpenoids found in essential oils, including thymol, carvacrol, eugenol, and menthol, are recognized for their strong antimicrobial activity against a variety of microorganisms (Husain et al., 2015; Kalemba & Kumato, 2003). Several research studies have proven the efficacy of various essential oils and their individual components in inhibiting *Salmonella* biofilms. In particular, sublethal levels of thyme oil, oregano oil, and carvacrol (at concentrations of 0.006-0.012%) inhibited *Salmonella* biofilm formation by 2 to 4 times, but they could not fully eradicate the biofilm (Soni et al., 2013).

Essential oils from *Origanum vulgare* (0.25%) and *Rosmarinus officinalis* (4%) successfully eradicated both young and mature biofilms of *Salmonella enteritidis* on stainless steel surfaces. These oils showed a time-dependent effect and employed a multi-target approach on the bacterial cell membrane (Lira et al., 2020). The essential oils of *Satureja montana* and *Thymus vulgaris* demonstrated strong antibiofilm, anti-adhesive, and bactericidal effects against *Salmonella typhimurium* and 12 *Salmonella* strains isolated from food (Miladi et al., 2016). Čabarkapa et al. (2019) examined the antimicrobial and antibiofilm properties of essential oils from *Origanum heracleoticum*, *O. vulgare, T. vulgaris,* and *T. serpyllum* against *S. enteritidis*. Their research indicated that these oils, along with their principal components (carvacrol and thymol), were effective in preventing biofilm formation at sub-MIC concentrations and eliminating 48-hour preformed biofilms in a dose-dependent manner.

Additionally, cinnamaldehyde, the main component of cinnamon bark EO (55-76%), at 2000 ppm/kg, Reduced the initial biofilm populations (7-7.5 log CFU/cm²) formed by *Salmonella* isolates from a conventional pig farm environment by a 6 log CFU reduction (Keelara et al., 2016).

Moreover, nisin Z has proven effective in providing protection against both Gram-positive *S. aureus* and Gram-negative *Salmonella enterica* sv. The authors suggest that nisin Z modulates the host immune system by utilizing mechanisms similar to those of natural human host defense peptides, involving various signal transduction pathways and growth factor receptors (Kindrachuk et al., 2013).

The antibacterial effects of essential oils, tested using the disk diffusion assay (DDA), are outlined in Table 1. Essential oils from thyme, cinnamon, rosemary, cumin, garlic, bay, black pepper, lemon, parsley, and nutmeg formed inhibition zones larger than 10 mm. Conversely, oils from orange, basil, and tarragon showed inhibition zones of 10 mm or less and were therefore considered non-inhibitory (García-Díez et al., 2016).

Table 1. The inhibitory effect of essential oils on *Salmonella* spp.

Essential oil	Thyme	Bay	Black papper	Cinnamon	Cumin	Garlic	Lemon	Nutmeg	Parsley	Rosemary
Salmonella spp	29.1 ± 9.5	12.5 ± 1.4	NI	13.7 ± 1.0	NI	10.5 ± 1.6	NI	NI	NI	13.4 ± 3.8

The inhibition zones were calculated, including the disk diameter of 6 mm.
NI indicates non-inhibitory.
Data are shown as mean (mm) ± standard deviation.
The essential oils of basil, orange, and tarragon are excluded due to no inhibition being observed.

Current research is therefore focused on the development of advanced delivery technologies, such as nanoemulsions and antimicrobial active films, to increase the stability and manage the controlled release of these compounds within food products (Ribeiro-Santos et al., 2017; Sharma et al., 2022). The incorporation of bioactive compounds as antimicrobial agents represents a promising alternative for improving food safety and controlling *Salmonella* contamination in the meat industry.

CONCLUSIONS

In conclusion, the use of bioactive compounds from natural sources presents a promising solution to improve the safety and quality of meat, given their ability to control Salmonella contamination and prevent spoilage. Bioactive compounds, such as polyphenols, terpenoids, and antimicrobial peptides, have demonstrated significant effects against *Salmonella* by inhibiting bacterial growth and biofilm formation. Additionally, these compounds help preserve the sensory and nutritional characteristics of meat, protecting it from oxidation and rancidity.

Case studies and experimental results support the efficacy from essential oils and different plant extracts in combating *Salmonella*, positioning them as viable and natural alternatives to traditional preservation methods that may affect meat quality. However, to optimize the application of these compounds in the meat industry, further research on their bioavailability, stability, and interactions with food matrices is essential to ensure maximum efficiency without negatively affecting the organoleptic characteristics of the products.

Finally, bioactive compounds offer significant potential to enhance food safety and improve meat quality while reducing the risks of microbiological contamination, such as *Salmonella*. Their adoption in a sustainable and well-regulated manner could transform the food industry, providing consumers with safer and more natural products.

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ANALYSIS OF A FUNCTIONAL PRODUCT FROM CARP (*Cyprinus carpio*) WASTE IN THE CONTEXT OF THE CIRCULAR ECONOMY

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Abstract

According to the data provided by the representative organization of the Romanian aquaculture sector, a production of 10,000 tons of fish is estimated for 2024. The fish processing industry is known for its high quantities of by-products, and in these circumstances, it is necessary to adopt the most correct approach to their disposal or recycling to prevent the challenges related to environmental management. The circular economy has been of increasing interest in recent years as the exploitation of fish waste represents a sustainable strategy for realizing a circular bio-economy by producing high-value-added compounds. This study aimed to obtain a concentrated soup from fish waste (bony skeleton, head, and skin) and to incorporate this concentrate into a paste obtained from processed fillets. Determinations were carried out to establish the gross chemical composition, color, and pH of the experimental batches, as well as the acceptability of this product to potential consumers.

Key words*: circular economy, concentrated soup, fish waste, value-added product.*

INTRODUCTION

With the global population expected to reach a staggering 8.5 billion people by 2030, there is a continuing concern about the need for food, and aquaculture is showing the highest growth globally.

The Food and Agriculture Organization of the United Nations (FAO) estimates that by 2030, aquaculture will account for 2/3 of all fish and seafood production (Venslauskas et al., 2021). Although fish is a quite popular food choice, globally, 7.2-12 million tons of fish waste is discarded annually, according to data presented by Manjudevi et al. (2024).

Increasing consumer demand for healthy food has initiated large-scale research and development of new products in the food industry (Anchidin et al., 2023). Adopting a more sustainable lifestyle requires the implementation of new methods capable of solving the problems related to the management of by-products resulting from fish processing. Thus, to meet industrial requirements as well as actions regarding environmental protection, different approaches have been conceived, among which the circular economy, being defined as "an economic system that replaces the end-of-life concept by reducing, reusing, recycling and recovering materials in production processes", (Fraga-Corral et al., 2022). Also, in recent years, more and more trends are pursuing the use of animal by-products as an integrated part in the realization of renewable material, which can then be used in different industries.

The search for new functional food ingredients from natural sources is one of the most important discussions in food science and technology (Olaniran et al., 2024).

More than 70% of all fish undergoes further processing before being placed on the market, resulting in the production of large quantities of between 20 and 80% of overproduced fish, depending on the level of processing (Coppola et al., 2021).

The by-products and waste resulting from fish processing are separated into by-products from eviscerating fish (gastrointestinal mass) and by-products from skinning, slicing, and filleting fish, resulting in by-products such as scales, skeletal remains, head, and fins.

According to statistics, the head represents between 9 and 12%, the viscera between 12

and 18%, the skin between 1 and 3%, the bones between 9 and 15% and the scales about 5% of the total weight of the whole fish (Olaniran et al., 2024).

The increase in meat production and consumption is due, in addition to the need to provide food for the ever-growing population, to its high nutritional and biological value, especially as a good source of complete proteins, with meat traditionally being associated with good health and prosperity (Ciobanu et al., 2025).

The meat we consume is an important component of the human diet. It contains essential nutrients that help to maintain normal physiological functions, improve immunity, and prevent certain diseases, including malnutrition (Ciobanu et al., 2023).

A fish-based diet has a positive nutritional impact, playing an important role in correcting unbalanced diets. As well as being an important source of high-quality protein containing all essential amino acids, fish provides essential fats (long-chain omega-3 fatty acids), vitamins (D, A and B), and minerals (Ca, I, Zn, Fe and Se) (Coppola et al., 2021).

Due to its chemical composition, but also its high consumption demand, fish accounts for about 17% of the animal protein intake of the global population and 6.7% of all protein consumed worldwide. Collagen is the most common structural protein found in the extracellular matrix of animal organisms.

It has been found that there are at least 29 distinct forms of collagen derived from animal tissues, each with its protein structure (Olaniran et al., 2024). Thus, fish by-products can be an excellent source in functional food ingredients manufacturing. With its unique sensory profile along with other qualities, meat and meat products play a significant role in human nutrition. The consumption of meat and meat products is increasing in parallel with the level of development and standard of living of society (Boișteanu et al., 2025).

MATERIALS AND METHODS

The current research was carried out at the Iasi University of Life Sciences, within the Department of Food Technologies.

The realization of the experimental samples was carried out in the Meat Processing and Processing Section, and the subsequent determinations were carried out in the Meat and Meat Preparations Laboratory and the Sensory Analysis Laboratory.

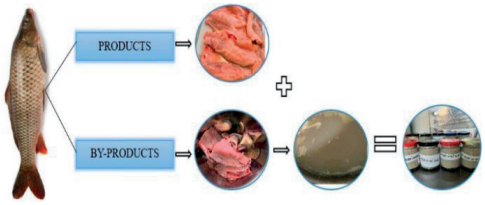


Figure 1. Methodology for obtaining experimental samples (original)

The biological material used consisted of fresh aquaculture carp and was purchased from a local supermarket under refrigerated conditions (2-4ºC) for 2 hours, and then subjected to a first stage of splitting, removing the gastrointestinal mass, the second stage consisting of slicing the fish, removing the head, tail and lateral fins and then a final filleting stage removing the biologically valuable tissue from the skeletal structure.

The bony skeleton and the head were used to produce the concentrated fish bone soup.

The super pods were boiled for 4 hours for the most efficient extraction of collagen and other proteins until a concentrated liquid was obtained, and the mass of sub pods began to crumble.

The resulting broth was filtered and stored under refrigerated conditions for 24 hours to gel.

The remaining carp fillets were heat-treated for pasteurization as shown in Table 1.

Table 1. Heat treatment applied to fish fillets

	Drying I	Smoking	Boiling	Drying II
Time (minutes)	20	30		10
Cell temperature (ºC)	45	60	76	72
Core temperature (ºC)	50	55	72	69
Moisture (%)	10	10	99	10

To make the fish paste with concentrated bone broth, the heat-treated skin-on fish fillets were minced using a Volf machine with a 3 mm diameter sieve and then finely ground using a Cutter (Cutter Titane 45 V) at 3.500 rpm until a fine homogeneous paste was obtained. The resulting emulsion was blended with three different percentages of fish bone broth (10%, 15%, and 20%), the pre-weighed spice mix was added, and the composition was homogenized using a blender (La Felsinea). The resulting samples were filled into 220 mL glass jars and subjected to a sterilization heat treatment for 2 hours at 98-110°C in a pressure boiling kettle. These technological processes resulted in 4 experimental batches of fish paste, a control samples without the addition of concentrated bone soup, and three batches containing 10%, 15%, and 20% concentrated soup. Subsequently, the experimental samples were analyzed from physico-chemical and sensory points of view. The physico-chemical analysis consisted of pH determination using a digital pH meter for meat (Hanna HI98163) fitted with an FC2323 electrode. Texture determinations were carried out with the TA1Plus texture analyzer, applying tests for the determination of TPA parameters, using a Kramer shear cell. The color characteristics were determined using the Konica Minolta Chroma Meter CR-410, which employs a spectrophotometric measurement technology using CIE color coordinates (L*, a*, b*). The gross chemical composition was determined by using a Food-Check analyzer, which uses a non-invasive technology based on infrared spectrometry to determine the percentages of moisture, protein, collagen, and fat. A panel of 50 semi-experienced panelists (students in their fourth year of study, food industry profile), with an average age of 21 years, was formed to evaluate sensory characteristics. The panelists received the questionnaire in digital format (Google Forms) to centralize the data as accurately as possible, and they were instructed on the sample samples received and the content of the questionnaire. A hedonic test (9-point scale) was applied, evaluating the general appearance, smell, taste, color, and consistency of the experimental samples. Score 1 represents the expression "extremely unpleasant" and score 9 the expression "extremely pleasant".

RESULTS AND DISCUSSIONS

To compare the differences between the means of the experimental samples in the analysis of physico-chemical determinations, the ANOVA statistical method was used, using the one-factor ANOVA test followed by the Tukey HSD test. From the analysis carried out it can be seen, as shown in Table 2, that in terms of fat content of the samples analyzed, significant variations were evident between all pairs of groups ($p<0.05$), the control sample showed the highest fat content (10.2%) while the values decreased with increasing percentage of added concentrated soup. Compared to the control sample, the samples with added concentrated soup showed lower fat concentration values, with the addition of soup significantly decreasing the fat, especially in the samples with 15% and 20% added concentrated soup. As regards the moisture content of the samples, the p-value ($p<0.05$) showed statistically highly significant differences, with a significant increase in the moisture content of the samples with added concentrated soup compared to the control sample. This increase was mainly due to the amount of moisture contained in the concentrated soup. The percentages of protein and collagen did not differ significantly, as shown by the p-values ($p>0.05$) of the two indices measured, the values being almost equal between all groups. The average protein content values were very close between the samples, ranging from 20.22% in the control sample to 20.24% in the 10% added sample. The most significant mean difference was observed between the control (SM) lot (20.22%) and the L3 lot (20.32%). The mean percentages of collagen in the total mass of the sample showed slight variations in the mean percentage between sample L2 (18.4%) and sample L3 (18.6%). This high proportion of collagen in the total protein is due to the high content of skin that was not removed from the surface of the fillets used. In the literature, the collagen content in fish varies according to the species, age, and method of analysis. For example, according to the study conducted by Duan et al. (2009), the collagen extracted from crap fish was 41.3%/dw for skin and about 40%/dw for bones compared to other species. This would correspond in our study to a content

of 12.7% collagen/dw. Thus, the values obtained in this study show that the high percentage of collagen results from the collagen found in the bone soup, but also from the collagen found in the skin and flesh of the fish.

Table 2. Physico-chemical composition of the experimental samples

Experimental groups		Fat (%)	Moisture (%)	Protein (%)	Collagen (%)
SM	**Count**	5	5	5	5
	Mean±SD	10.02^{b}±0.16	69.24^{a}±0.20	20.22^{a}±0.19	18.46^{a}±0.25
	Min.-Max.	9.74-10.29	69.10-69.37	20.07-20.36	18.27-18.64
L1	**Count**	5	5	5	5
	Mean±SD	9.64^{b}±0.24	69.62^{b}±0.13	20.24^{a}±0.16	18.48^{a}±0.16
	Min.-Max.	9.36-9.91	69.48-69.75	20.09-20.38	18.29-18.66
L2	**Count**	5	5	5	5
	Mean±SD	8.40^{a}±0.40	70.26^{c}±0.11	20.4^{a}±0.12	18.4^{a}±0.15
	Min.-Max.	8.12-8.68	70.12-70.39	20.25-20.54	18.21-18.58
L3	**Count**	5	5	5	5
	Mean±SD	8.62^{a}±0.31	70.14^{c}±0.11	20.32^{a}±0.13	18.6^{a}±0.17
	Min.-Max.	8.34-8.89	70.00-70.27	20.17-20.46	18.41-18.78
p-value		2.87884E-07**	1.76414E-08*	0.28^{ns}	0.43^{ns}

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$). SM - sample control, L1 - 10% concentrated soup L2 - 15% concentrated soup, L3 - 20% concentrated soup.

The mean values of the color parameters of the experimental samples show that the mean values of the brightness vary between 66.42 and 68.81, with a slight increase as the percentage of added concentrated soup increases. The red-green color component (a*) shows mean values ranging between 4.68 (L3) and 5.52c (L2), with sample L2 showing a slight increasing trend compared to the control sample, having a darker shade of the lot color, while lots L1 and L3 show a decreasing trend in color concentration. The yellow-blue color component (b*) shows mean values between 15.7a and 16.33c. However, there is a general trend of increasing b* value with increasing percentage of concentrated bone broth, suggesting an intensification of the yellow color tone The ANOVA results showed significant differences between groups for the parameters a* and b*, suggesting that the addition of concentrated bone broth significantly influences the color of the fish paste (p<0.05), with implications for visual perception and consumer acceptability of the product. One-factor ANOVA statistical analysis revealed significant differences in pH variation between the samples analyzed. The differences between the control sample and the samples with added concentrated soup are statistically significant (p<0.05), the pH evolution being altered due to the contribution of weakly acidic organic compounds or mineral salts present in the concentrated soup. The decrease in average values in the samples with added soup may have a positive influence on the microbiological stability of the product, increasing its shelf life. By correlating the results presented in Table 3, an inversely proportional relationship can be observed between color intensity and pH parameters. It can be observed that as the pH value decreases, the color intensity of the samples increases. pH may be a factor that can influence the color variations of the studied samples.

As for the textural profile analysis, significant differences were found between the control sample and the samples to which concentrated soup was added, according to the p-values (p<0.05), as shown in Table 4.

In terms of the hardness of the samples, it can be observed that the impact of the addition of concentrated soup from bones decreased the consistency of the samples, this parameter decreasing significantly from a mean value of 29.49 N in the control sample to a mean value of 16.85 N in the L3 sample.

Table 3. Physical parameters of the experimental samples

Experimental groups		L*	a*	b*	pH
SM	**Count**	5	5	5	5
	Mean±SD	66.42^{a}±0.24	5.12^{a}±0.07	15.70^{a}±0.06	6.67^{a}±0.02
	Min.-Max.	66.15-66.77	5.06-5.25	15.61-15.76	66.64-6.7
L1	**Count**	5	5	5	5
	Mean±SD	66.53^{a}±0.49	4.89^{b}±0.14	15.98^{b}±0.11	6.61^{b}±0.01
	Min.-Max.	65.66-66.91	4.65-5.02	15.86-16.16	6.59-6.63
L2	**Count**	5	5	5	5
	Mean±SD	67.71^{ab}±1.09	5.52^{c}±0.06	15.91^{ab}±0.20	6.58^{a}±0.01
	Min.-Max.	65.87-68.73	5.45-5.61	15.56-16.07	6.57-6.6
L3	**Count**	5	5	5	5
	Mean±SD	68.81^{b}±0.91	4.68^{d}±0.09	16.33^{c}±0.10	6.55^{b}±0.03
	Min.-Max.	67.71-70.19	4.58-4.78	16.26-16.51	6.51-658
p-value		0.00	4.06E-09	1.58E-05	3.2E-06

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$), SM - sample control, L1 - 10% concentrated soup, L2 - 15% concentrated soup, L3 - 20% concentrated soup.

Table 4. Textural parameters of experimental samples

Experimental groups		Hardness (N)	Adhesiveness (N x mm)	Springiness	Cohesiveness (N)	Gumminess (N)	Chewiness (J)
SM	**Count**	5	5	5	5	5	5
	Mean±SD	29.49^{d}±1.36	53.20^{d}±0.51	0.27^{d}±0.01	1.66^{d}±0.05	49.77^{b}±3.96	13.46^{b}±1.50
	Min.-Max.	28.47-32.14	52.69-53.99	0.26-0.28	1.60-1.73	45.59-55.74	11.85-15.60
L1	**Count**	5	5	5	5	5	5
	Mean±SD	26.52^{c}±1.24	4.53^{b}±0.40	0.24^{c}±0.01	0.52^{a}±0.05	13.96^{a}±1.94	3.36^{a}±0.60
	Min.-Max.	25.27-28.58	3.89-4.82	0.23-0.25	0.47-0.58	12.40-16.60	2.85-4.15
L2	**Count**	5	5	5	5	5	5
	Mean±SD	23.60^{b}±0.78	3.60^{a}±0.43	0.20^{b}±0.008	0.50^{a}±0.06	12.04^{a}±1.74	2.50^{a}±0.39
	Min.-Max.	22.17-24.47	3.38-4.37	0.2-0.22	0.40-057	9.28-14.02	1.85-2.94
L3	**Count**	5	5	5	5	5	5
	Mean±SD	16.85^{a}±0.99	6.28^{c}±0.03	0.17^{a}±0.008	0.81^{c}±0.06	13.81^{a}±1.79	2.45^{a}±0.32
	Min.-Max.	15.84-18.46	6.22-6.31	0.17-0.19	0.73-088	11.71-16.17	2.10-2.91
p-value		2.9048E-11	1.22461E-28	2.43506E-10	3.26292E-15	9.14E-14	7.12067E-13

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$), SM - sample control, L1 - 10% concentrated soup, L2 - 15% concentrated soup, L3 - 20% concentrated soup.

This is basically due to the increase in the amount of gelling components in the realized products. In terms of adhesion, it can be seen that although the mean values decrease in the 10% and 15% of the control sample, respectively, they increase in L3, which indicates a recovery of the binding properties due to the amount of collagen. However, fish concentrated soup additions affect the ability of the dough to return to its original shape after elastic deformation, as the samples analysed show lower values in the ados (0.17) than in the control (0.27). Internal consistency demonstrates that at high concentrations of concentrated soup addition, collagenous compounds contribute to the stabilization of the product. The results show that, although compared to the mean value of the control sample (1.66 N), the mean values decrease in L1 (0.52 N) and L2 (0.50 N), in L3 the mean value increases to 0.81 N. The foaminess and chewiness were also significantly influenced by the concentrated soup addition ($p<0.05$). The gumonized parameter showed low mean values, from 49.77 N in the control sample to 13.81 N in the 20%-added sample. Chewability showing values of the means from 13.56 J in the control sample to 2.45 J in L3,

demonstrating a reduction in the mechanical forces required for product fragmentation and chewing.

According to Pogurschi et al. (2018), consumption behavior in a "Western" society has changed in terms of the type of food consumed, but it is still limited in relation to income level and food price. However, according to Ciobanu et al. (2024) consumers turn their attention to a clean label, still being guided by the sensory properties that the final product presents. As regards the experimental samples conducted, the sensory analysis revealed variations in the visual appearance and color of the samples studied. Batch L2 (15%) recorded the highest mean value, 8.96, which explains that this addition brings an improvement to the visual impression. In the case of color, there is an increase in the averages up to batch L2 (15%), then a sharp decrease in sample L3 (20%), which shows that the concentrated bone soup added in a higher percentage changes the pigmentation of the color of the product, making it more unattractive. The mean values did not vary significantly in terms of taste, with a slight decrease in batches L2 and L3, which may indicate a change caused by mineral compounds that negatively modified the taste profile. In samples with a high percentage of concentrated soup, the panelists also did not appreciate the odor, with low average values. Consistency was the most stable attribute, with similar values for the lots. From a statistical point of view, the *p*-value > 0.05, indicating that none of the differences observed between the experimental samples were statistically significant.

Table 5. Sensory parameters of the experimental samples

Experimental groups		Overall Appearance	Consistency	Taste	Smell	Color
SM	**Mean±SD**	8.54±2.21	7.68±2.25	5.85±2.92	7.36±2.16	6.28±1.75
L1	**Mean±SD**	8.66±1.89	7.56±2.34	5.98±2.89	6.34±2.11	7.05±1.46
L2	**Mean±SD**	8.96±1.94	7.62±2.37	5.16±3.03	5.26±2.30	7.36±1.61
L3	**Mean±SD**	8±2	7.68±2.54	5.24±3.05	5.4±2.30	3.28±1.52
p-value		0.600151	0.993281	0.917887	0.989985	0.804889

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$), SM - sample control, L1 - 10% concentrated soup, L2 - 15% concentrated soup, L3 - 20% concentrated soup.

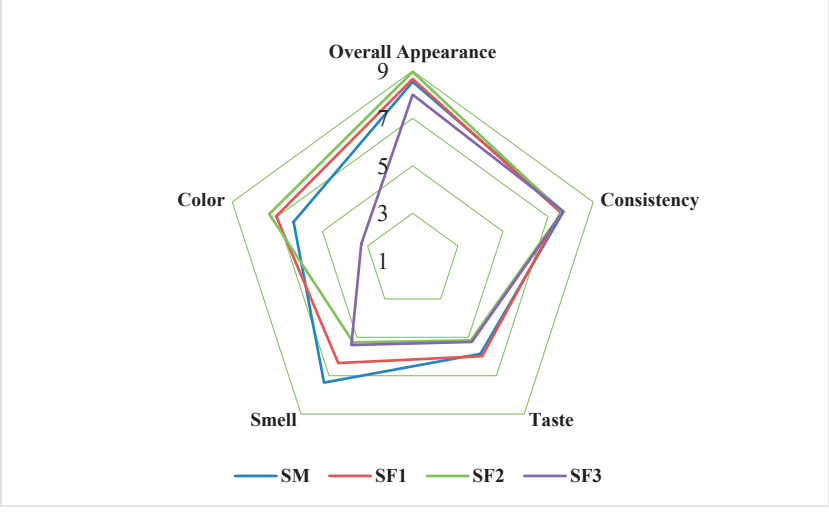


Figure 2. Diagram of the results of the sensory analysis of the experimental samples

The radar plot gives an overview of the values presented in the statistical table. Thus, from the diagram, it is confirmed that the experimental samples have a high score for general appearance, with L2 (SF2) in evidence. Overlapping lines are highlighted in the case of

consistency, which denotes that there is uniformity among the experimental samples. Taste is affected by the percentages of soup added, which corresponds to the values in Table 5. At the same time, the downward trend of the L2 (SF2) and L3 (SF3) samples compared to the control sample is also observed. In the case of color, the diagram clearly shows a downward trend of the values presented in the statistical table for the experimental sample with 20% added bone broth.

CONCLUSIONS

The results obtained in this study showed that the addition of concentrated bone soup significantly influenced both the physico-chemical properties and the sensory profile of the experimental batches. Lot L2 (15%) provided a favorable balance between improved nutritional parameters and acceptable sensory characteristics. Therefore, the use of concentrated soups in moderate quantities may present a viable technological strategy for the valorization of animal-origin soups and the development of innovative food products with added value and improved functional profile.

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THE USE OF CARROT JUICE AS AN AGENT TO IMPROVE THE QUALITY ATTRIBUTES OF A PRODUCT OBTAINED FROM CHICKEN BREAST (*Musculus pectoralis*)

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Abstract

The increasing demand for the consumption of chicken meat products imposes the need for continuous and complex research in this application area. Thus, healthier alternatives are being sought by developing preparations with bioactive compounds from natural sources in their structure to improve their intrinsic properties. The current study focused on the injection of carrot juice in different percentages (5%, 10%, and 15%) in three experimental groups, using the anatomical region of the Musculus pectoralis. Experimental batches were subjected to an enzymatic wet aging process using vacuum wet aging under refrigerated conditions, followed by heat treatment. Following the analysis of the data obtained, significant results were recorded. Carrot juice can be considered as a bioactive component in optimizing the overall quality of the finished product, bringing changes in both the nutrient profile, physico-chemical indicators, and sensory properties.

Key words*: bioactive compounds, carrot juice, meat product, quality.*

INTRODUCTION

The meat industry sector is considered one of the most important players in the global industry, and innovation in this sector is driven by consumer demand and increasing competitive pressure. Chicken meat products are among the top consumer preferences because there are no religious or cultural restrictions on chicken meat (Boișteanu et al., 2025). At the same time, according to Pogurschi et al. (2018), in Romania, consumers' perceptions of food have changed rapidly in response to socio-economic dynamics over the last three decades.

According to Statista (n.d.), *Production of meat worldwide 1990-2024* (Retrieved December 3, 2024) in the year 2024, globally, poultry meat production exceeded pork production by 17.03 million metric tons, reaching a total of 138.75 million metric tons.

The poultry meat consumption market is experiencing accelerated growth. It is projected to expand by 65% from 2015 to 2035, outpacing the growth rates of pork (by 35%) and eggs (by 50%). Poultry meat consumption meets the nutritional standards of a contemporary balanced diet: high in protein, low in calories, fat, and cholesterol, and with excellent digestive bioavailability (Wołoszyn et al., 2020; Goluch et al., 2023). Since meat is an indispensable source of nutrients in the human diet (Ciobanu et al., 2005), there has been a growing concern about healthy nutrition in recent years. Thus, the food industry needs to pay more attention to optimizing the quality characteristics of meat products and converting them into functional foods, capitalizing on the benefits of products of plant origin and those of animal origin (Anchidin et al., 2024).

Although functional food is considered to be an emerging field, the concept of functional food was first defined in 1980 in Japan, and it refers to a processed food that contains ingredients that, in addition to their nutritional value, also support certain body functions (Cencic et al., 2010). In the U.S. regulatory framework, Health Canada characterized functional foods in 1995 as products "similar in appearance to conventional foods that are consumed as part of a regular diet and that have demonstrated physiological benefits and/or reduce the risk of

chronic disease beyond basic nutritional functions" (DeFelice, 1995). In 1996, FUFODSE (European Commission on Functional Food Science in Europe) was initiated at the European level, actively involving many leading European nutrition and related sciences experts, and coordinated by the International Life Sciences Institute (ILSI) Europe. This commission aimed to reach a consensus on "scientific concepts of functional foods". Thus, in 1999, the functional food was defined: "a food can be considered functional if it is shown to beneficially influence one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to improving health and well-being and/or reducing the risk of disease. A functional food must remain a food and manifest its effects in amounts normally consumed: it is not a pill or a capsule but part of the normal diet" (Diplock et al., 2000).

The development of functional foods has seen significant expansion across most food categories; however, their implementation is not evenly distributed across the whole food industry, with notable variations between different market segments (Kotilainen et al., 2006; Menrad, 2003). Thus, in the context of an accelerated development of functional foods, with varying applicability across food industry segments, the meat sector, especially poultry, shows considerable potential for functional innovation. Poultry meat is distinguished by several processing-friendly technological characteristics, such as neutral flavor, uniform texture, and light color, which facilitate the adaptation of products to market requirements. These properties make it possible to formulate products with varied sensory profiles according to the preferences of target consumer groups (e.g., products with intense flavors for adults or moderately flavored variants for children) (Barbut, 2012). As there is an increasing demand for functional and healthy foods, integrating non-animal-derived compounds is an effective measure to increase the quality of meat products (Ciobanu et al., 2025). Thus, both the involvement of various food quality enhancers (Zugravu et al., 2017) and a thorough assessment of the consequences of the use of additives or other compounds on sensory properties, which influence consumption behaviours, are necessary for modern food processing (Ciobanu et al., 2024).

The present study aimed to develop a chicken meat product by injecting the breast with carrot juice in proportions of 5%, 10%, and 15%, respectively, and to analyse the physico-chemical parameters and sensory properties.

MATERIALS AND METHODS

To achieve the objective of this study, four experimental batches were realized with chicken breast (*Musculus pectoralis*) as biological material. A control sample was injected with a saline mixture obtained from water and salt at a 1.2% concentration, and the other three experimental samples were injected with different carrot juices (5%, 10%, 15%) and salt (1.2%). The chicken breast used as raw material was purchased from a local supermarket. The carrots used for the new product were purchased at the local market and then processed into carrot juice using an electric juicer (Myria MY4013) with cold-pressing technology.

The four experimental samples are presented in Table 1, together with the ingredients used in the manufacturing process and the heat treatments applied. All experimental stages, including batch preparation and analysis, were carried out at the Ion Ionescu de la Brad University of Life Sciences in Iasi, in the specialized sections and laboratories for the processing and analysis of meat products - the Meat Processing and Processing Section and the Meat Products Control Laboratory.

Table 1. Composition of the experimental samples

Samples	Ingredients (%)		
	Chicken breast	Carrot juice	Salt
CS	98.8	-	1.2
L1CJ5%	93.8	5	1.2
L2CJ10%	88.8	10	1.2
L3CJ15%	83.8	15	1.2

CS - Control sample; L1CJ5% - 5% carrot juice; L2CJ10% - 10% carrot juice; L3CJ15% - 15% carrot juice.

The experimental protocol involved preparing the raw material (chicken breast) and its injection with the obtained salting solutions using a semi-automatic injection device. After injection, the samples were vacuum-packed and subjected to wet maturation for 24 h at refrigeration temperature (0-4°C). After the wet

maturation step, the samples were removed from the vacuum and individually placed on stainless steel sticks, which were subjected to the heat treatment. The heat-treatment regime applied to the samples was structured in several stages, including drying, smoking, and boiling, respecting specific temperature and time parameters for each phase. Details of the process are presented in Table 2.

Table 2. The applied head treatment

Heat treatment stage	Time (min)	Cell temperature (°C)	Core temperature (°C)	Moisture (%)
Drying I	15	65	55	25
Smoking	30	65	55	25
Boiling	-	76	72	99
Drying II	20	80	72	25

After obtaining the four samples of chicken breast injected with the salting solutions, the samples were subjected to physicochemical analysis following heat treatments.

A FoodCheck analyzer (Bruins Instruments, OmegAnalyzer, Germany), a spectrophotometer using infrared light beams, was used to determine the gross chemical composition. Moreover, it quantitatively analyzes the protein, moisture, collagen, and salt content using infrared radiation to determine the organic composition of the samples analyzed (Ciobanu et al., 2023; Anchidin et al., 2023).

Physical analysis of the samples obtained included texture, colour and pH analysis. To determine textural parameters, we used the Lloyd Instruments TA1Plus (Ametek, UK) texturometer, equipped with a force sensor capable of measuring up to 500 N and a Warner-Bratzler knife. The colorimetric analyses of the studied lots were performed using the Konica Minolta Chroma Meter CR-410 (Konica Minolta, Osaka, Japan) portable colorimeter through the CIE Lab color system, which quantifies color parameters L* (black to white), a* (green to red) and b* (blue to yellow) using the standard illuminant D65. Before analysis, the colorimeter was calibrated using a standard white calibration plate. For each sample, five measurements were made, both surface and cross-sectional.

The pH analysis was performed using a Hanna Instruments portable pH meter, model HI99163, taking 5 measurements for each sample at different points of the sample, taking into account the pH variations in correlation with temperature.

Data distribution analysis was performed using SPSS Statistics, version 26.0 (IBM Corp., 2019). A comparison of mean values was performed using a one-way analysis of variance (ANOVA), followed by the Tukey post-hoc test. Differences between means were considered significant at a significance level of $p < 0.05$.

RESULTS AND DISCUSSIONS

Table 3 shows the main physico-chemical characteristics analyzed in the studied samples (fat, moisture, collagen protein, and salt).

Table 3. Arithmetic mean ± standard deviation of the physicochemical parameters determined for the analysed samples

Physicochemical parameters					
	%				
Samples	**Fat**	**Moisture**	**Protein**	**Collagen**	**Salt**
CS	2.12±0.044^{c}	75.78±0.044^{a}	21.84±0.089^{a}	20.26±0.114^{a}	1.92±0.044^{c}
L1CJ5%	1.68±0.083^{b}	76.14±0.054^{b}	22.06±0.151^{b}	20.38±0.044^{a}	1.9±0.1^{c}
L2CJ10%	1±0.070^{a}	76.74±0.05^{c}	22.08±0.044^{b}	20.52±0.837^{ab}	0.88±0.044^{a}
L3CJ15%	0.94±0.054^{a}	76.76±0.054^{c}	22.12±0.044^{b}	20.54±0.054^{b}	1.28±0.044^{b}
p value	4.67E-15	2.12E-15	0.00097775	0.00011	1.70E-14

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); CS - Control sample; L1CJ5% - 5% carrot juice; L2CJ10% - 10% carrot juice; L3CJ15% - 15% carrot juice.

The results presented in Table 3 show statistically significant differences ($p \leq 0.05$) between the analyzed samples for all physico-chemical parameters. As expected, the gradual incorporation of carrot juice visibly influenced the final composition of the product.

The fat content of the analyzed samples showed highly significant differences ($p \leq 0.001$) between the experimental groups. The progressive addition of carrot juice strongly influenced the lipid profile of the chicken breast product. The control sample had the highest fat content (2.12±0.044), followed by a significant decrease in L1CJ5% (1.68±0.08) and L2CJ10% (1±0.07), with the minimum value in L3CJ15% (0.94±0.054). This decreasing trend reflects a dilution effect caused by carrot juice's aqueous composition, which partially replaces native lipids during injection.

Moisture content increased slightly with increasing percentage of added carrot juice, from 75.78±0.044 in the control to 76.76±0.054 in the L3CJ15%. These results suggest a water-holding capacity-enhancing effect, possibly due to water and polysaccharides in the carrot juice matrix.

Protein values increased moderately with the addition of carrot juice, from 21.84±0.089 in the control to 22.12 ± 0.044 in L3CJ15%. This increase can be attributed to its rich content in bioactive compounds with functional roles, including carotenoids: α-carotenoids: α-carotene, β-carotene, lutein, zeaxanthin and lycopene (Bystrická et al., 2015), phenolic acids (chlorogenic, ferulic, p-coumaric and caffeic acids) (Ahmad et al., 2019), as well as oxyanthracocyanins including sinapoylglucosyl-galactoside, cyanidin-3-O-xylozyl-(feruloylglucosyl)-galactoside and cyanidin-3-O-xylozyl-(coumaroylglucosyl)-galactoside (Sharma et al., 2012) that participate in optimizing the nutritional value of the product. Collagen content remained relatively constant, ranging from 20.26±0.114 in the control to 20.54±0.054 in L3CJ15%. The differences, although statistically significant, are minor, suggesting that carrot juice does not significantly affect the connective protein structure of chicken breast.

The salt parameter value showed a clear decrease between the control and L3CJ15%, from 1.92±0.044 to 1.28±0.044. This decrease can be explained by the dilution effect of the added carrot juice, which positively contributes to the reformulation of low-sodium products.

According to Table 4, the addition of carrot juice in different concentrations did not cause statistically significant ($p > 0.05$) changes in color parameters on the surface of reformulated chicken breast-based products compared to color parameters in the product section, where statistically significant differences ($p \leq 0.05$) were revealed between experimental batches. This indicates that carrot juice had a more intense effect on the product section's color profile than on the product surface. In terms of colorimetry at the surface of the chicken breast injected with carrot juice, the values for lightness (L)* varied slightly, between 52.00±4.345 for L1CJ5% and 55.27±4.72 for L3CJ15%, while the control sample recorded 54.306±2.046. Although statistically insignificant, these variations can be attributed to the natural pigments in the carrot juice (mainly carotenoids), which can influence the surface reflectance without significantly altering the perceived brightness. For parameter a* (degree of red color), values remained statistically similar between groups, ranging from 15.692±1.370 to 17.818±0.673. This trend indicates that injecting the carrot juice, although rich in orange-red pigments, did not significantly emphasize the red component on the surface. Parameter b* (degree of yellow color) showed close values in all batches (between 30.778±1.620 and 34.852±3.953), suggesting a slight visually perceived yellow tint, but without statistical significance. In color analysis of chicken breast sections injected with carrot juice, the lightness (L*) values decreased significantly in the reformulated samples, from 78.428±1.299 in the control to 72.416±1.894 in L3CJ15%. This decrease may be due to the migration of carrot juice pigments, which resulted in a slight darkening of the internal tissue. A lower L* value indicates reduced brightness and more intense color.

The parameter a* (degree of red color) increased directly to the percentage of carrot juice, from 5.658±0.302 in the control sample to 11.03±2.611 in L3CJ15%. This increase confirms that carrot juice's carotenoid pigments

penetrated the muscle structure, significantly emphasizing the reddish hues. According to Anchidin et al. (2023), this does not negatively influence the analyzed products, as a reddish color is specific to meat products and helps to increase their attractiveness. The b* (degree of yellow color) values followed the same trend, rising from 12.206±0.207 in the control sample to 18.482±1.129 in L3CJ15%. These data emphasize the influence of the salting mixture with carrot juice on the coloration, giving the product a more intense yellow-orange hue in the section.

Table 4. The color results obtained from the analyzed samples of the reformulated product

Samples	L*(D65)	a*(D65)	b*(D65)
Surface color of chicken breast injected with carrot juice			
CSS	54.306±2.046^{a}	16.288±1.094^{a}	31.942±2.554^{a}
L1CJ5%S	52±4.345^{a}	17.05±1.172^{a}	30.778±1.620^{a}
L2CJ10%S	53.844±1.048^{a}	17.818±0.673^{ab}	34.682±2.796^{a}
L3CJ15%S	55.27±4.72^{a}	15.692±1.370^{a}	34.852±3.953^{a}
p value	0.507157	0.040817	0.095175
Color in section of chicken breast injected with carrot juice			
CSSe	78.428±1.299^{b}	5.658±0.302^{a}	12.206±0.207^{a}
L1CJ5%Se	73.826±0.822^{a}	8.534±0.226^{b}	12.716±0.391^{a}
L2CJ10%Se	73.022±2.444^{a}	9.42±0.830^{b}	13.108±1.072^{a}
L3CJ15%Se	72.416±1.894^{a}	11.03±2.611^{b}	18.482±1.129^{b}
p value	0.000182	0.000132	3.38E-09

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); CSS - Control sample at surface; L1CJ5%S - 5% carrot juice at surface; L2CJ10%S - 10% carrot juice at surface; L3CJ15%S - 15% carrot juice at surface; CSSe - Control sample in section; L1CJ5%Se - 5% carrot juice in section; L2CJ10%Se - 10% carrot juice in section; L3CJ15%Se - 15% carrot juice in section.

According to Table 5, the instrumental texture analysis revealed statistically significant differences ($p \leq 0.05$) between the analyzed samples for the hardness and mechanical working parameters.

Hardness values varied significantly with carrot juice concentration. The control sample recorded a value of 14.44±1.63, and the highest value was observed in L1CJ5% (16.43±3.53), suggesting that a 5% carrot juice level may contribute to strengthening the protein network. In contrast, the higher levels of carrot juice (L2CJJ10% and L3CJ15%) led to a considerable shrinkage of the product, with the hardness recording reduced values (5.77±0.74 - L2CJ10% and 7.61±2.25 - L3CJ15%). This decrease in the values of the hardness parameter can be explained by the action of enzymes or natural acids in the juice, which may interfere with the gelation process of myofibrillar proteins during heat treatment. The values for the mechanical work or energy required to cut the carrot juice-injected chicken breast followed a similar pattern, ranging from 153.29±1.83 in the control to 184.05±1.83 in L1CJ5%, then dropping sharply to 105.14±2.72 in L2CJ10% and 114.35±2.10 in L3CJ15%. These results reflect the mechanical resistance opposed during chewing or slicing. The initial increase in L1CJ5% suggests a structural hardening, possibly due to protein-carbohydrate interactions, while the decrease at higher concentrations again indicates a weakening of the matrix. In conclusion, although the moderate addition of carrot juice (5%) improved firmness and cut strength, higher amounts compromised the product's structural integrity. This behavior suggests the need to optimize juice levels to balance technological performance with nutritional benefits.

Table 5. Evaluation of the textural parameters of the analyzed samples of the reformulated product

Samples	Textural parameters	
	Hardness (N)	Work of cutting (N mm)
CS	14.4387503±1.630 [b]	153.28681±1.828 [c]
L1CJ5%	16.4252034±3.530 [b]	184.0468473±1.830 [d]
L2CJ10%	5.7693771±0.736174 [a]	105.14256±2.722 [a]
L3CJ15%	7.61318±2.25153 [a]	114.346±2.095 [b]
p value	1.10E-07	2.60E-24

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); CS - Control sample; L1CJ5% - 5% carrot juice; L2CJ10% - 10% carrot juice; L3CJ15% - 15% carrot juice.

The pH variation at different temperatures (15.18-18.72°C) for the analyzed samples is shown in Figure 1. No statistically significant differences (p>0.05) were observed between the control and experimental samples injected with carrot juice. The control sample recorded a value of 6.28±0.008, while L1CJ5%, L2CJ10%, and L3CJ15% had values of 6.25±0.043, 6.27±0.069, 6.34±0.075, respectively. The consistency of these results indicates that injecting the carrot juice did not significantly alter the acid-base equilibrium of the chicken meat product. Even though there was a slight tendency for the pH values to increase in direct proportion to the increasing concentration of the carrot juice, the differences remained within narrow limits, reflecting the stability of the protein and enzyme systems involved in the post-processing phases.

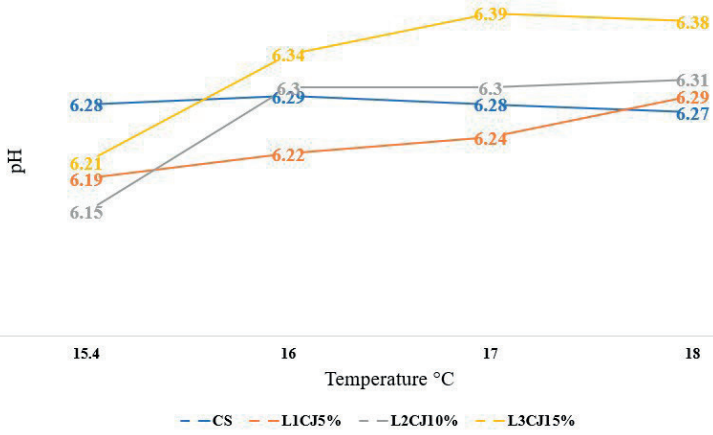


Figure 1. The pH variation at different temperatures (15.18-18.72°C) for the control samples (CS) and the experimental variants (L1CJ5%, L2CJ10%, L3CJ15%)

CONCLUSIONS

The results of this study show that including carrot juice as an injection agent in chicken breast to obtain reformulated products leads to measurable and partially significant changes in the quality attributes of the final product. From a nutritional point of view, using the salting mix with carrot juice contributed to a moderate increase in protein and moisture content while reducing the salt level, a beneficial effect in formulating healthier products.

The color analysis revealed that, although the surface parameters remained relatively unchanged, the product's cross-section underwent visible changes, with red and yellow shades intensifying due to the migration of carotenoids. These color changes may improve consumers' perception of the product's natural and fresh character.

The textural profile showed a concentration-dependent effect: at low levels (5%), carrot juice increased firmness and resistance to slicing, while higher concentrations (10%,

15%) led to a significant destabilization of the structure, possibly influenced by the enzymatic or acidic action of the compounds in the salting mixture with carrot juice.

The pH values remained constant in all experimental variants, and the inclusion of carrot juice did not disturb the matrix's acid-base balance, indicating good technological compatibility.

Overall, carrot juice can be considered a natural ingredient favorable to the development of functional chicken products, especially when used in moderate concentrations. It contributes positively to nutritional value, visual appearance, and texture modulation while supporting clean-label innovation and sustainable processing strategies.

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THE USE OF HONEY AS A BIOACTIVE AGENT FOR OPTIMIZING THE BEEF MATURATION PROCESS AND THE IMPACT ON THE SENSORY PROPERTIES OF THE FINAL PRODUCT

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Abstract

In human health concerns and diet diversification, food science research focuses on developing functional products, using sustainable and health-promoting methods. Beef, an important source of nutrients, is often rejected due to its sensory characteristics and tough texture, which is a challenge for consumers with dental problems. This study aimed to evaluate the effectiveness of honey as a bioactive agent in optimizing the beef maturation process, considering its beneficial effects on health and the environment. Three different concentrations of honey (10%, 20%, and 30%) applied in the wet-aging process for 48 hours were tested, followed by a thermal process and a detailed sensory evaluation. The use of honey as a natural bioactive agent in the wet-aging process of beef significantly improves meat quality, both in terms of physicochemical and sensory attributes, in a dose-dependent manner, without affecting internal color stability, thus offering benefits for consumer acceptability and the development of functional and sustainable food products.

Key words*: beef, honey, maturation process, quality.*

INTRODUCTION

Beef is valued for its high protein content of high biological value, its significant intake of heme iron and zinc, and its rich sensory profile, defined by its intense umami taste and firm texture. However, it is often perceived to be tougher to digest, particularly in collagen-rich muscle pieces (Scollan et al., 2006; Boișteanu et al., 2024a). The addition of honey as a bioactive agent can enhance the marbling and tenderness of beef during the maturation process (Ciobanu et al., 2024). Research indicates that honey, when used in marinades, can significantly improve meat tenderness by affecting protein structures and enhancing flavor profiles. Honey contains enzymes and acids that can break down proteins, leading to increased tenderness in beef. Studies show that marinades with honey resulted in a notable reduction in hardness measurements of beef compared to control samples (Istrati et al., 2012; Istrati et al., 2015). The presence of honey in marinades also contributes to the hydrolysis of collagen and myofibrillar proteins, which are crucial for meat tenderness (Istrati et al., 2012). Marbling, or intramuscular fat (IMF), is essential for meat quality (Boișteanu et al., 2024b). Honey's nutritional components may influence adipogenic activity, promoting fat deposition in muscle tissues (Wandita et al., 2018). The combination of honey with other ingredients in marinades can enhance the overall flavor and moisture retention, which indirectly supports the development of marbling during the maturation process (Istrati et al., 2015). While honey shows promise in enhancing beef quality, it is essential to consider that excessive sweetness or improper ratios in marinades could potentially mask the natural flavors of the meat, leading to a less desirable product (Ciobanu et al., 2024). While feed management technologies play a critical role in minimizing environmental contaminants such as mineral oil hydrocarbons (MOH) at the farm level, post-harvest interventions, such as the use of natural bioactive agents like honey during beef maturation, offer complementary strategies to enhance both food safety and quality throughout the production chain (Matei et al., 2024). In beef sausage, concentrations of 5.0% to 7.5% honey were associated with improved sensory attributes, including color, aroma, and

texture, while also enhancing safety and storability (Mohammed et al., 2013).

For beef jerky, a concentration of 24% honey yielded optimal quality, affecting pH, moisture, and protein content positively (Karlina et al., 2022). This concentration was found to produce a desirable balance of flavor and texture. While higher concentrations of honey can enhance certain sensory properties, they may also lead to undesirable sweetness or color changes, as noted in some studies (Tolon et al., 2000). Thus, the optimal concentration should be tailored to the specific product and consumer preferences (Zugravu et al., 2017; Ciobanu et al., 2025; Manoliu et al., 2024).

This study aimed to assess the potential of honey as a bioactive agent for improving the beef maturation process, taking into account its health and environmental benefits. Three honey concentrations (10%, 20%, and 30%) were applied during a 48-hour wet-aging treatment, followed by thermal processing and comprehensive sensory evaluation.

MATERIALS AND METHODS

In accordance with the experimental protocol, three experimental samples were prepared with different honey additions (10%, 20%, and 30%), alongside a control sample, all subjected to a 48-hour wet-aging process aimed at improving the physicochemical and sensory parameters of the final product. The honey used in the experiment was sourced from a local producer holding the relevant quality certifications. To avoid potential conflicts of interest, the commercial identity of the supplier was kept anonymous.

The selection of these biocomponents aimed to simulate a realistic scenario, in line with applicable European regulations, such as Regulation (EC) No. 178/2002 on food safety, Regulation (EU) No. 1169/2011 on consumer information, and other relevant legal provisions in the field of food and natural product safety. The raw material (beef) was purchased from the local market and met the quality and traceability standards set by European regulations, including Regulation (EC) No. 854/2004 and Regulation (EU) No. 1169/2011. The experimental setup was designed in full compliance with these regulations. Beef muscle samples were treated with honey at concentrations of 10%, 20%, and 30% during the wet-aging process, which was carried out in embossed vacuum bags consisting of two layers: an inner layer of 60 µm polyethylene suitable for food contact, and an outer layer of 15 µm polyamide with UV filter. Sealing was performed using an ATM Machinery vacuum device (chamber power: 630 W), and the samples were stored at 2°C, protected from light, throughout the 48-hour aging period (Boișteanu et al., 2024a).

The influence of honey addition on the beef aging process was assessed by analyzing the colorimetric profile, textural properties, and pH at 24 and 48 hours. Color characteristics were measured using a Chroma Meter MINOLTA, model CR-410 (Konica Minolta, Osaka, Japan), according to the CIE Lab color system. The expressed color parameters were L* (lightness), a* (green-red component), and b* (blue-yellow component). The device was calibrated using a standard white reference plate, and CIELAB values were recorded at three distinct points on each sample.

Texture was assessed using a Lloyd Instruments TA1Plus texture analyzer (AMETEK, UK), equipped with a 500 N load cell. Tests were conducted at a constant speed of 100 mm/min, with an initial extension of 90 mm, controlled by software version 4.1.5.999 and embedded version 2.0.300. pH values were measured using a portable pH meter, Hanna Instruments, model HI99163. For each sample batch, five measurements were taken at different points to ensure data representativeness. After 48 hours of wet-aging, the samples were subjected to thermal treatments as detailed in Table 1. Subsequently, the experimental samples were analyzed for their moisture, protein, collagen, fat, and salt content using a versatile near-infrared (NIR) spectroscopic method, according to the protocol described by Gucianu et al. (2024). The analyses were performed using the Food Check meat analyzer (Bruins Instruments, Germany). For the sensory evaluation, the samples were cut into uniform pieces, anonymized through coding, and distributed to the tasting panel. The evaluation was carried out by a panel of 47 semi-trained participants (students and academic staff from the “Ion

Ionescu de la Brad" University of Life Sciences in Iași), aged between 20 and 42 years, in accordance with ISO 8586:2023. The participants assessed the four experimental variants using a 9-point hedonic scale, where a score of 1 corresponded to "dislike extremely" and a score of 9 to "like extremely". The evaluation was conducted based on six sensory attributes: texture, taste, odor, color, cross-sectional appearance, and overall appearance (Boișteanu et al., 2025). The distribution of data was assessed using IBM SPSS Statistics version 26.0 (IBM Corp., 2019). Statistical comparisons were conducted through one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, using IBM SPSS Statistics version 21. A significance threshold of $p < .05$ was applied for all analyses.

Table 1. The applied head tratament

Heat treatment stage	Time	Temperature inside the cell	Temperature in the thermal centre	Humidity
	minutes	°C	°C	%
Drying I	15	65	55	10
Smoking	30	65	55	10
Boiling	-	74	72	99
Drying II	20	80	72	10

RESULTS AND DISCUSSIONS

According to Table 2 pH analysis revealed a concentration-dependent modulation of acidification during wet-aging. While the control sample exhibited a significant decrease in pH over 48 hours ($p \leq 0.05$), honey-treated samples showed a more stabilized profile, particularly at 30% concentration, suggesting a potential antimicrobial and buffering effect of honey during maturation.

Table 2. pH variation of samples (SM, SH1, SH2, SH3) from 24 to 48 hours

Sample	Ph	
	24 h	48 h
SM	5.826 ± 0.037^{cA}	5.672 ± 0.063^{aB}
SH1	5.8 ± 0.036^{cA}	5.784 ± 0.100^{aA}
SH2	5.71 ± 0.054^{bA}	5.634 ± 0.027^{abB}
SH3	5.6 ± 0.018^{aA}	5.614 ± 0.088^{abA}

Different superscript letters (a, b, c, d) within the same column and (A, B, C, D, E, F) within the same row indicate statistically significant differences, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); SM - sample control, SH1 - 10% honey; SH2 - 20% honey; SH3 - 30% honey.

Control (SM) has the largest pH drop, which suggests uncontrolled natural maturation with potential risk of accelerated spoilage. Treatment with 10% and 20% honey (SH1, SH2) attenuates the pH drop and has a protective effect, but without completely inhibiting maturation. SH3 (30% honey) has the lowest initial value, but it does not decrease afterwards, which is why honey in high concentration can reduce microbiological activity, stabilizing the product. Statistically significant differences ($p \leq 0.05$) confirm the significant influence of honey concentration on the pH evolution over time. Textural parameters such as Hardness, Work of Cutting, and Tensile Strength showed significant variations depending on both the honey concentration and the maturation time (Table 3).

Table 3. Evolution of textural parameters during beef maturation with different honey concentrations (Hardness, Work of Cutting, Tensile Strength)

Sample	Hardness (N)		Work of Cutting (Nmm)		Tensile Strength (MPa)	
	24 h	48 h	24 h	48 h	24 h	48 h
SM	13.71 ± 0.401^{aA}	20.35 ± 0.269^{aB}	136.37 ± 2.093^{aC}	345.23 ± 2.399^{aD}	0.20 ± 0.072^{aF}	0.15 ± 0.044^{aE}
SH1	20.95 ± 0.351^{bA}	23.44 ± 0.450^{bB}	234.87 ± 3.694^{bC}	382.93 ± 2.004^{bD}	0.11 ± 0.038^{aE}	0.19 ± 0.036^{aF}
SH2	23.20 ± 0.168^{cA}	25.77 ± 0.453^{cB}	255.73 ± 3.385^{cC}	434.34 ± 1.696^{cD}	0.25 ± 0.119^{bF}	0.16 ± 0.065^{aE}
SH3	25.37 ± 0.159^{dA}	28.27 ± 0.489^{dB}	345.87 ± 2.111^{dC}	466.88 ± 1.485^{dD}	0.18 ± 0.058^{abF}	0.13 ± 0.027^{aE}

Different superscript letters (a, b, c, d) within the same column and (A, B, C, D, E, F) within the same row indicate statistically significant differences, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); SM - sample control, SH1 - 10% honey; SH2 - 20% honey; SH3 - 30% honey.

After 48 hours of wet-aging, all samples treated with honey (SH1, SH2, SH3) exhibited significantly higher hardness values compared to the control (SM), indicating a firmer and more compact texture. The most notable increase was observed in SH3 (30% honey), suggesting that higher concentrations may promote protein cross-linking or dehydration effects, possibly enhanced during the subsequent thermal treatment. Similarly, the Work of Cutting increased progressively with honey concentration, reflecting greater resistance to mechanical breakdown.

This may be attributed to the interaction between honey constituents, such as sugars and phenolic compounds, and muscle proteins, which could contribute to the structural reinforcement of the meat matrix.

Tensile Strength showed a more variable trend: while SH2 reached the highest value at 24 h, a decrease was noted at 48 h, possibly due to enzymatic degradation over time.

These findings support the hypothesis that honey not only serves as a bioactive agent but also plays a role in modulating meat texture during maturation, offering new possibilities for customizing meat products based on target consumer preferences.

The color parameters of the beef surface (L, a, b*) were significantly influenced by both the honey concentration and the maturation time (Table 4).

The lightness value (L*) increased in all samples from 24 to 48 hours, with the highest values observed in SH3 (30% honey), suggesting that higher honey concentrations may enhance light scattering on the meat surface, possibly due to surface dehydration.

The control sample (SM) showed the lowest L* values, confirming that honey addition contributes to a lighter appearance of the meat over time.

Table 4. Surface color parameters during beef maturation (24-48 h) with varying honey concentrations (10%, 20%, and 30%)

Sample	Surface					
	L*(D65)		a*(D65)		b*(D65)	
	24 h	48 h	24 h	48 h	24 h	48 h
SM	31.376 ± 0.261^{aA}	32.062 ± 0.662^{aB}	17.016 ± 0.298^{bC}	17.434 ± 0.332^{bC}	3.58 ± 0.065^{aD}	3.608 ± 0.380^{aD}
SH1	34.868 ± 0.491^{bA}	35.358 ± 3.858^{aB}	17.298 ± 2.149^{bC}	16.748 ± 1.811^{bD}	6.058 ± 1.973^{bE}	3.392 ± 2.810^{aF}
SH2	33.87 ± 0.786^{bA}	35.036 ± 1.544^{aB}	12.866 ± 0.780^{aD}	11.646 ± 1.128^{aC}	4.628 ± 0.617^{bF}	3.974 ± 1.151^{aE}
SH3	34.418 ± 1.254^{bA}	36.78 ± 0.784^{bB}	13.77 ± 1.065^{aC}	13.42 ± 1.195^{aC}	5.678 ± 1.396^{bF}	3.798 ± 1.054^{aE}

Different superscript letters (a, b, c, d) within the same column and (A, B, C, D, E, F) within the same row indicate statistically significant differences, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); SM - sample control, SH1 - 10% honey; SH2 - 20% honey; SH3 - 30% honey.

The parameter a*(D65) showed distinct behavior, while SM and SH1 maintained higher values at both time points, SH2 and SH3 displayed significantly lower redness levels. This could be due to partial pigment degradation or antioxidant interactions between honey phenolics and myoglobin, reducing oxymyoglobin stability in higher honey concentrations. Interestingly, SH1 maintained stable a* values between 24-48 h, indicating moderate honey levels may preserve red coloration better. Regarding the parameter b*(D65), values were highest at 24 h in SH1 and SH3, suggesting early-stage honey-protein interactions or pigment contributions from honey itself. However, a general decrease in b* was noted at 48 h in all honey-treated groups, potentially due to pigment oxidation or moisture migration affecting surface reflectance. These findings underline the role of honey not only as a bioactive agent for texture enhancement, but also as a modulator of visual quality. From a consumer perspective, brighter and more uniform meat color may improve product appeal, particularly in populations sensitive to meat freshness indicators. Section color parameters (L, a, b*) of beef sections showed limited but noteworthy variation depending on honey concentration and maturation time (Table 5). The lightness values (L*) remained statistically unchanged across all samples and time points, indicating that honey application did not significantly affect internal light reflection or water distribution in the muscle matrix after 48 hours of wet-aging and thermal treatment. This stability in L* suggests that the maturation process did not cause notable discoloration or internal browning. The control sample (SM) experienced an increase in a*(D65) from 24 to 48 hours, consistent with typical oxygenation and blooming of myoglobin in non-treated beef. However, honey-treated samples (SH1-SH3) demonstrated a decline in a* values over time, especially in SH3 (30% honey). This may reflect the antioxidant activity of honey, which

can limit oxymyoglobin formation and promote metmyoglobin stabilization, leading to reduced red intensity in the meat interior. The slight but significant reductions in a* at higher honey concentrations may also suggest deeper interactions between phenolic compounds and muscle pigments (Anchidin et al., 2023). From a consumer standpoint, this suggests that the honey-assisted maturation process preserves the internal visual quality of beef, which is particularly important for cooked or sliced products where interior appearance is relevant (Ciobanu et al., 2023a). Overall, these results highlight that while surface color is more susceptible to changes from honey addition and exposure to oxygen, sectional color remains more stable, with subtle pigment interactions becoming evident primarily in redness values. The parameter b*(D65) remained relatively stable across all treatments and time points, with no significant differences. This suggests that the wet-aging process with honey did not alter the chromatic balance between red and yellow tons in the inner meat, supporting the visual homogeneity of the final product.

Table 5. Section color parameters during beef maturation (24-48 h) with varying honey concentrations (10%, 20%, and 30%)

Sample	Section					
	L*(D65)		a*(D65)		b*(D65)	
	24 h	48 h	24 h	48 h	24 h	48 h
SM	31.931 ± 0.386^{aA}	31.991 ± 0.366^{aA}	18.492 ± 0.822^{bB}	19.056 ± 1.312^{cC}	4.142 ± 0.787^{aD}	4.792 ± 1.747^{aD}
SH1	33.392 ± 2.665^{aA}	33.382 ± 2.665^{aA}	19.648 ± 1.656^{bD}	17.84 ± 0.507^{abcC}	4.566 ± 0.988^{aE}	4.11 ± 0.664^{aE}
SH2	32.962 ± 0.618^{aA}	32.952 ± 0.618^{aA}	17.562 ± 0.829^{abD}	16.73 ± 0.851^{aC}	3.78 ± 0.642^{aE}	3.668 ± 0.674^{aE}
SH3	33.912 ± 1.475^{aA}	33.900 ± 1.475^{aA}	17.856 ± 0.659^{bC}	16.186 ± 0.605^{aB}	4.324 ± 0.538^{aF}	3.936 ± 0.418^{aE}

Different superscript letters (a, b, c, d) within the same column and (A, B, C, D, E, F) within the same row indicate statistically significant differences, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); SM - sample control, SH1 - 10% honey; SH2 - 20% honey; SH3 - 30% honey.

The physicochemical profile of thermally treated beef samples revealed significant variations influenced by the concentration of honey applied during wet-aging (Table 6). The protein content showed a clear trend, with the highest value recorded in SH2 (20% honey, 21.10%), followed by the control sample (SM), SH1 (10%), and the lowest in SH3 (30%). This indicates that moderate honey concentrations (20%) may help preserve or even slightly enhance protein integrity, possibly due to reduced proteolysis and water loss during thermal processing. Conversely, the lower protein percentage in SH3 could be attributed to higher fat content and reduced moisture, leading to relative protein dilution.

Table 6. Physicochemical parameters (Protein %, Moisture %, Fat %, Lipid %) determined for the analysed batches after thermal treatment

Sample	Protein, %	Moisture, %	Fat, %	Lipid, %
SM	20.84 ± 0.054^{c}	72.18 ± 0.192^{c}	6.42 ± 0.228^{b}	19.12 ± 0.109^{c}
SH1	20.46 ± 0.056^{b}	71.18 ± 0.589^{b}	7.78 ± 0.637^{c}	18.74 ± 0.089^{b}
SH2	21.1 ± 0.070^{d}	73.06 ± 0.114^{d}	5.4 ± 0.123^{a}	19.46 ± 0.054^{d}
SH3	19.84 ± 0.054^{a}	68.48 ± 0.268^{a}	10.98 ± 0.130^{d}	18.06 ± 0.114^{a}

Different superscript letters (a, b, c, d) within the same column indicate statistically significant differences, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); SM - sample control, SH1 - 10% honey; SH2 - 20% honey; SH3 - 30% honey.

Moisture content followed a similar trend, with SH2 exhibiting the highest value (73.06%), while SH3 had the lowest (68.48%). The higher moisture retention in SH2 could suggest a protective effect of the 20% honey matrix against water loss during cooking, possibly by forming a light coating that reduced evaporation. In contrast, the drop in moisture at 30% honey may reflect excessive sugar-induced protein crosslinking, leading to firmer structure and more water expulsion. Fat and lipid contents varied significantly. SH3 exhibited the highest fat (10.98%) and the lowest lipid percentage (18.06%), while SH2

had the lowest fat content (5.4%) and the highest lipid retention (19.46%). These differences suggest that honey concentration influences the distribution and release of intramuscular fat during thermal treatment. SH2 may promote better emulsification and stabilization of lipids during cooking, while SH3's higher fat value might reflect less efficient lipid retention and a more compact, fat-rich matrix. The optimal honey concentration appears to be 20% (SH2), balancing protein preservation, moisture retention, and lipid stability during cooking. In contrast, excessive honey (30%) may negatively affect physicochemical properties by altering water and protein dynamics, despite increasing fat retention. These findings support the use of honey not only for sensory improvement but also for modulating the nutritional and technological properties of beef products. Sensory evaluation results (Table 7; Figure 1) revealed a generally positive impact of honey addition on the organoleptic quality of beef samples, as evidenced by the improved scores for appearance, color, texture, and taste in all honey-treated variants compared to the control. The enhancements suggest that honey not only contributes to flavor development but may also positively influence texture and visual appeal, making the final product more acceptable and appealing to consumers.

Table 7. Sensory attributes assessed for the beef samples following treatment

Sample	Overall Appearance	Section Layout	Color	Texture	Smell	Taste
SM	7.31±2.475^{a}	6.74±2.471^{a}	7.74±2.471^{a}	7.72±2.570^{a}	7.96±2.268^{a}	7.54±2.421^{a}
SH1	7.80±2.544^{a}	7.16±2.341^{b}	8.06±2.341^{b}	8.84±2.607^{b}	8.44±2.517^{b}	8.81±2.685^{b}
SH2	8.21±2.589^{b}	7.56±2.252^{b}	8.46±2.252^{b}	8.96±2.724^{b}	8.28±2.322^{b}	8.13±2.780^{b}
SH3	7.91±2.336^{a}	7.95±2.130^{b}	8.79±2.130^{b}	8.56±2.598^{b}	7.08±2.296^{a}	8.88±2.560^{b}

Different superscript letters (a, b, c, d) within the same column indicate statistically significant differences, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$); SM - sample control, SH1 - 10% honey; SH2 - 20% honey; SH3 - 30% honey.

Sensory evaluation results indicate that the addition of honey in the beef maturation process significantly influenced most sensory attributes ($p \leq 0.05$), especially texture, color and taste. Compared to the control (SM), all honey-treated samples (SH1, SH2, SH3) recorded significantly higher scores for color, texture, and taste, showing a perceptible improvement in product acceptability. Sample SH2 (20% honey) had the highest scores for overall appearance (8.21 ± 2.589) and texture (8.96 ± 2.724), suggesting an optimal combination of honey concentration and sensory profile. SH3 (30% honey) stood out with the highest score for color (8.79 ± 2.130) and taste (8.88 ± 2.560), although it had a lower value for smell (7.08 ± 2.296), which may suggest a more intense or atypical aromatic impact. The control sample (SM) obtained the lowest scores for all attributes, confirming the potential of honey as a bioactive agent to improve the sensory characteristics of matured meat.

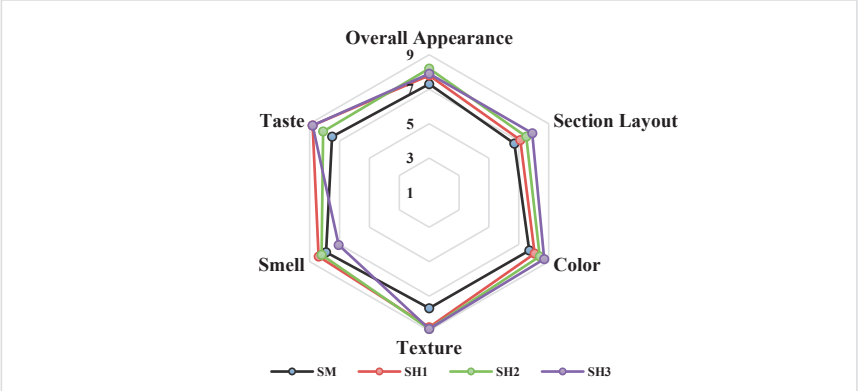


Figure 1. Graphical representation of sensory attribute results for the analyzed samples

The control sample (SM) recorded the lowest scores across all evaluated sensory attributes, including appearance, section layout, color, texture, smell, and taste. This outcome highlights the limited sensory appeal of beef that has not undergone enhancement through natural bioactive agents. In contrast, the samples treated with honey, particularly those with higher concentrations (20% and 30%), demonstrated significantly improved scores, suggesting a notable enhancement in consumer-perceived quality. These findings support the hypothesis that honey, due to its natural bioactive compounds, can positively influence the maturation process, leading to improved organoleptic properties. The consistent increase in sensory scores across treated samples underscores honey's potential as a sustainable and health-promoting alternative for optimizing meat products (Ciobanu et al., 2023b).

CONCLUSIONS

The results of this study demonstrate that honey, used as a natural bioactive agent in the beef wet-aging process, exerts a significant and multifunctional influence on meat quality. Its incorporation during maturation led to measurable improvements in physicochemical parameters, such as protein retention, moisture content, and fat balance, while enhancing sensory attributes like texture, color, taste, and overall appearance. These improvements were most pronounced at higher concentrations (20-30%), suggesting a dose-dependent response where the bioactive components in honey actively interact with muscle proteins and lipids. Importantly, despite the surface-level changes in brightness and chromatic values, internal color stability was preserved, indicating that the structural integrity and oxidative balance of the meat matrix were not adversely affected by honey exposure. From a consumer acceptability perspective, the improved tenderness and flavor, combined with a visually more appealing product, offer clear advantages for market diversification, particularly for vulnerable groups such as the elderly or individuals with masticatory difficulties. Consequently, honey-assisted maturation not only aligns with the growing demand for clean-label and functional foods but also provides a sustainable and health-conscious approach to enhancing meat processing technologies.

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SUSTAINABLE STRATEGIES FOR THE USE OF ANIMAL BY-PRODUCTS IN MEAT PRODUCTS WITH HETEROGENEOUS STRUCTURE: APPROACHES TO COMBAT FOOD WASTE

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Abstract

Food waste is a major problem that has negative effects on the environment and the global economy. In recent years, research has focused on promoting food circularity and sustainability. Animal by-products, with a valuable nutritional profile, offer significant potential to replace synthetic additives. This study examines the integration of beef fat from the meat industry bone by-products into heterogeneous meat products. Various fat proportions (2%, 4% and 6%) were investigated to assess the impact on the overall quality. Although some segments of the population reject fat-added meat products due to health concerns, the research aims to identify sustainable solutions that are both environmentally and health beneficial, thus contributing to a significant reduction in food waste.

Key words*: by-products, food waste, meat products.*

INTRODUCTION

Meat by-products are rich in bioactive peptides and functional ingredients, which can be valorized to improve meat product formulations (Boişteanu et al., 2025). Addressing regulatory and safety barriers is important for the effective utilization of these by-products, promoting sustainability in the meat industry, and in light of recent research on the impact of feed management technologies on mineral oil hydrocarbons (MOH) contamination, which highlights the importance of on-farm control measures to reduce risks and ensure food safety throughout the production chain (Gagaoua et al., 2024; Matei et al., 2024). The meat supply chain experiences substantial losses, with 20% of meat intended for consumption wasted (Pinto et al., 2022). By-products often end up in landfills or incineration, contributing to environmental pollution (Mohan & Long, 2021). Valorization of these by-products can lead to sustainable practices, reducing overall food waste and enhancing resource efficiency (Pinto et al., 2022; Mohan & Long, 2021; Gucianu et al., 2024). While the valorization of animal by-products presents numerous benefits, challenges remain in implementing these processes effectively across the meat supply chain (Gucianu et al., 2024). The potential for innovation in this area is significant, yet it requires careful management to ensure safety and sustainability (Boişteanu et al., 2024). Animal by-products, due to their rich nutritional profile, hold substantial potential as natural alternatives to synthetic additives (Zugravu et al., 2017). The sustainable strategies for utilizing animal by-products in meat products focus on valorization, regulatory frameworks, and innovative processing techniques. These strategies aim to transform waste into valuable resources, thereby enhancing the sustainability of the meat industry while addressing environmental concerns (Anchidin et al., 2024). Meat by-products can be processed to extract bioactive peptides, which have health benefits and can be incorporated into functional foods (Gagaoua et al., 2024). The meat industry is increasingly turning by-products into marketable items, including high-value ingredients for food and non-food applications (Baldi et al., 2021; Mohan & Long, 2021). The evolution of the rendering industry has led to the production of safe, high-quality products from animal by-

products, contributing to a sustainable food chain (Woodgate, 2023). Innovations in processing techniques allow for the efficient conversion of by-products into valuable resources, such as biofuels and animal feeds, thus reducing waste (Mohan & Long, 2021; Jiang et al., 2020). Utilizing by-products can significantly lower greenhouse gas emissions associated with meat production, contributing to climate change mitigation (Jiang et al., 2020). While these strategies present significant opportunities for sustainability, challenges remain in consumer acceptance and the implementation of innovative technologies (Anchidin et al., 2024; Ciobanu et al., 2024; Ciobanu et al., 2025). Balancing economic viability with environmental responsibility is essential for the future of the meat industry.

MATERIALS AND METHODS

To achieve the objectives of the study, three samples with different percentages of fat (2%, 4%, 6%) resulting from the boiling heat treatment of bone by-products from the meat industry were realized. The fat obtained may represent a viable alternative to various synthetic components used to improve the final quality of food products. The raw material used was purchased on the local market and certified in accordance with European Union regulations on food safety and traceability of products of animal origin, including Regulations (EC) No 854/2004 and (EU) No 1169/2011. The experimental batches, together with the ingredients used and the heat treatments applied, are presented in Table 1. They were carried out in food processing and research workshops in compliance with the applicable regulations, including Regulations (EC) No 853/2004 on the hygiene of products of animal origin and (EC) No 854/2004 on official controls of products intended for human consumption, as well as other relevant food safety and hygienic processing regulations. The experimental samples involved the incorporation of fat obtained by boiling beef bone by-products to replace synthetic binding agents. The experimental samples include SF_1 - 2% fat introduced into the product; SF_2 - 4% fat introduced into the product; SF_3 - 6% fat introduced into the product, and the control sample, where no fat was introduced. The control sample and the experimental batches were subjected to the same heat treatment as shown in Table 1 and Table 2.

Table 1. Experimental batch formulation

Sample	Sample components			
	Raw material	Fat	Salt	Other ingredients
	%			
SM	96	-	2	2
SF_1	94	2	2	2
SF_2	92	4	2	2
SF_3	90	6	2	2

SM - sample control; SF1 - 2% fat; SF2 - 4% fat; SF3 - 6%

Table 2. The applied head tratament

Heat treatment stage	Time	Temperature inside the cell	Temperature in the thermal centre	Humidity
	minutes	°C	°C	%
Drying I	30	45	30	10
Smoking	40	55	40	10
Boiling	-	74	72	99
Drying II	10	80	72	10

The experimental samples were refrigerated for 24 hours and then subjected to laboratory analysis to evaluate the physicochemical profile. Gross chemical determinations included quantitative analysis of moisture, protein, collagen and salt content using near-infrared spectroscopy (NIR), a versatile and non-destructive method described by Gucianu et al. (2024). These determinations were performed using the Food Check meat analyser (Bruins Instruments, Germany). NIR spectroscopy refers to a region of the electromagnetic spectrum between 700 and 2500 nanometers, corresponding mostly to the colors red, orange, and yellow in the visible spectrum. In this range, various substances absorb and emit light, allowing significant interactions with biological materials and systems (Ciobanu et al., 2023).

The evaluation of the color characteristics of the samples was carried out using the MINOLTA Chroma Meter, model CR-410

(Konica Minolta, Osaka, Japan), according to the CIE Lab chromatic system. Parameters analyzed included L* (brightness: 0 - black, 100 - white), a* (positive values for red, negative for green) and b* (positive values for yellow, negative for blue). After calibrating the equipment with a white standard plate, CIELAB values were recorded for three samples. Color intensity (chroma) was determined by the formula: Chroma = $\sqrt{(a^2 + b^2)}$. The pH value was determined with a Hanna Instruments portable pH meter, model HI99163, by taking five measurements for each batch at different points of each sample, following the pH dynamics as a function of temperature. Texture parameters were analyzed using a Lloyd Instruments TA1Plus texturometer (AMETEK, UK), equipped with a force cell with a measuring capacity of up to 500 N. Testing was carried out at a constant speed of 100 mm/min with an initial extension of 90 mm. The equipment was operated using software version 4.1.5.999 and Embedded version 2.0.300. Data distribution was evaluated using SPSS Statistics software version 26.0 (IBM Corp., 2019). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test in IBM SPSS Statistics version 21. Differences were considered significant for p values < 05.

RESULTS AND DISCUSSIONS

According to the data presented in Table 3, the analyzed batches showed statistically significant differences. As expected, the fat content showed significant variations ($p < 0.05$) between the SM sample and SF_1, SF_2, and SF_3.

Table 3. Arithmetic mean ± standard deviation of the physicochemical parameters determined for the analysed batches

Physicochemical parameters					
%					
	Fat	Moisture	Protein	Collagen	Salt
SM	6.3±0.158^a	71.76±0.563^d	20.52±0.363^c	19.22±0.083^d	2.84±0.054^c
SF_1	8.62±0.311^b	70.42±0.311^c	20.36±0.054^c	18.64±0.054^c	2.64±0.134^b
SF_2	10.88±0.148^c	68.51±0.158^b	19.76±0.260^b	18.14±0.089^b	2.62±0.148^a
SF_3	14.02±0.083^d	65.74±0.421^a	19.14±0.089^a	17.36±0.054^a	2.62±0.044^a

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$). SM - sample control; SF_1 - 2% fat; SF_2 - 4% fat; SF_3 - 6% fat.

Fat incorporation was significant, influencing at the same time the other physico-chemical parameters of the product. This increase was accompanied by a statistically significant decrease in moisture content, indicating an inverse relationship between fat and water retention in the matrix. The dilution effect of the fat addition also contributed to a gradual decrease in protein and collagen concentrations, with the lowest values recorded in sample SF_3 (19.22% protein and 17.36% collagen, respectively). Salt content showed minimal variation, but significant differences were observed between samples, probably due to changes in water binding capacity and salt distribution in the modified matrix. These results suggest that the incorporation of rendered beef fat from bone by-products alters the overall composition of the product, with potential implications for both texture and nutritional profile. Despite the moderate protein reduction, the valorization of animal by-products contributes to a more sustainable production model and may offer functional benefits in terms of juiciness and palatability, provided consumer acceptance barriers are addressed (Manoliu et al., 2023).

The color parameters showed significantly influenced by the incorporation of beef fat obtained from bone by-products, as shown in Table 4. These changes highlight the impact of fat addition on the optical properties of the product, influencing consumer perception and overall appearance. Increased fat levels contributed to a brighter and more saturated color profile, which may enhance product appeal despite potential pigment dilution.

The brightness values L*(D65) were significantly increased in all reformulated samples (SF_1-SF_3) compared to the control (SM), with the highest value recorded in SF_1 (56.906), indicating a brighter appearance. This can be attributed to the presence of the added fat, which typically scatters light more

efficiently in the matrix, increasing the perceived brightness. The parameter a*(D65) showed a slight but significant decrease, especially in the SF_3 sample (13.69) compared to the control SM (14.11). This reduction suggests a myoglobin pigment dilution effect due to the inclusion of fat. However, the differences were marginal, and the overall red appearance of the product remained visually acceptable.

b*(D65) values increased significantly with fat level, especially in SF2 and SF3 (13.37 and 13.53, respectively), indicating a shift towards a warmer color hue. Similarly, Chroma (C*), which expresses color saturation, was significantly higher in all samples with added fat compared to the control. The highest chroma value was observed in SF_1 (19.36), suggesting a more vivid and intense color perception in the reformulated product.

Table 4. The color results obtained from the analyzed batches of the reformulated product

	L*(D65)	a*(D65)	b*(D65)	Chroma (C*)
SM	55.65±0.755^{a}	14.11±0.105^{b}	12.17±0.181^{a}	18.64±0.020^{b}
SF_1	56.90±0.365^{b}	14.02±0.060^{b}	13.34±0.149^{b}	19.36±0.027^{a}
SF_2	56.74±0.241^{b}	13.84±0.396^{ab}	13.37±0.551^{b}	19.25±0.025^{a}
SF_3	56.72±0.330^{b}	13.69±0.104^{a}	13.53±0.157^{b}	19.25±0.013^{a}

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$). SM - sample control; SF_1 - 2% fat; SF_2 - 4% fat; SF_3 - 6% fat

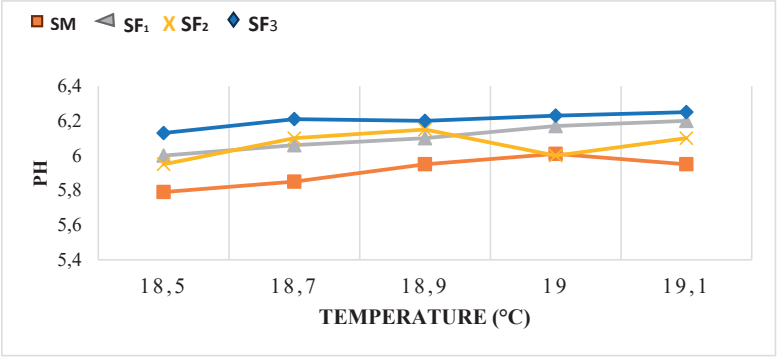


Figure 1. The pH variation at different temperatures (18.5-19.1 °C) for both the control samples (SM) and the experimental variants (SF_1, SF_2, SF_3)

Figure 1 illustrates the variation in pH at different temperatures (18.5-19.1°C) for the control samples (SM) and the reformulated ones (SF_1, SF_2, SF_3). All samples showed a general trend of increasing pH values with rising temperature. Notably, the sample with the highest fat content (SF_3) exhibited the most stable and highest pH values across the entire temperature range analyzed, suggesting a potential buffering effect provided by the added beef fat. In contrast, the control sample showed more pronounced pH fluctuations, which may indicate lower thermal resistance.

The incorporation of beef fat derived from bone by-products had a noticeable impact on the texture profile of the reformulated meat products in the absence of the edible membrane (Table 5). Among the analyzed parameters, hardness have results significantly from the control (SM: 10.61 N) to SF_1 (9.96 N). However, a slight decrease was observed at higher fat levels (SF_2 and SF_3), indicating a potential softening effect due to matrix restructuring at elevated fat concentrations.

Adhesiveness decreased progressively from the control to the SF3 sample, with statistically significant differences ($p \leq 0.05$), suggesting improved product handling and lower stickiness at higher fat levels.

This may be beneficial in terms of machinability and consumer acceptability. Springiness and cohesiveness were also affected. Springiness dropped from 0.36 (SM) to 0.17 (SF_3), while cohesiveness remained

relatively stable, except for a slight decrease in SF_2. These changes indicate a tendency toward a less elastic and more deformable texture as fat content increased, possibly due to the weakening of the protein network.

Table 5. Evaluation of the textural profile of analyzed batches without edible membrane

Sample	TPA (Texture Profile Analysis)					
	Hardness (N)	Adhesiveness (N x mm)	Springiness	Cohesiveness	Gumminess (N)	Chewiness (J)
Without membrane*						
SM	10.61 ± 0.564^{a}	50.77 ± 0.602^{c}	0.36 ± 0.018^{d}	0.19 ± 0.029^{b}	1.67 ± 0.355^{a}	0.60 ± 0.122^{b}
SF_1	9.96 ± 0.356^{b}	46.99 ± 1.313^{b}	0.30 ± 0.019^{c}	0.22 ± 0.030^{b}	2.39 ± 0.262^{ab}	0.72 ± 0.090^{b}
SF_2	8.76 ± 1.662^{b}	44.74 ± 2.508^{b}	0.22 ± 0.013^{b}	0.17 ± 0.031^{ab}	1.68 ± 0.517^{b}	0.37 ± 0.101^{a}
SF_3	8.47 ± 0.871^{b}	41.55 ± 0.992^{a}	0.17 ± 0.158^{a}	0.23 ± 0.025^{b}	2.17 ± 0.186^{a}	0.37 ± 0.060^{a}

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$). SM - sample control; SF_1 - 2% fat; SF_2 - 4% fat; SF_3 - 6% fat; *edible membrane (natural) with a diameter of 28-30 mm.

Gumminess and chewiness, which are derived parameters combining hardness, cohesiveness, and springiness, showed variable responses. While gumminess values remained in a narrow range with no consistent trend, chewiness decreased significantly in the SF2 and SF3 samples compared to the control, which might indicate a softer, less resistant structure during mastication. Overall, the textural modifications observed in fat-enriched samples reflect a balance between structural reinforcement (at low fat levels) and softening effects (at higher fat inclusion). These outcomes suggest that the inclusion of beef fat from bone by-products can be optimized to improve or tailor textural properties, depending on the target sensory profile and processing characteristics. Covering the samples with an edible membrane significantly altered the textural profile of the reformulated products, particularly in combination with the fat level introduced (Table 6). Hardness values were considerably higher in all membrane-coated samples compared to their counterparts without membrane (Table 5), indicating that the edible casing contributed to a firmer structure. However, a progressive decrease in hardness was observed with increasing fat content, from 22.11 N in the control (SM) to 14.22 N in SF_3 ($p \leq 0.05$), suggesting that higher fat inclusion softens the internal matrix despite the structural contribution of the membrane.

Table 6. Evaluation of the textural profile of analyzed batches covered with edible membrane

Sample	TPA (Texture Profile Analysis)					
	Hardness (N)	Adhesiveness (N x mm)	Springiness	Cohesiveness	Gumminess (N)	Chewiness (J)
With membrane*						
SM	22.11 ± 1.044^{b}	48.39 ± 0.880^{c}	0.41 ± 0.031^{d}	0.24 ± 0.110^{a}	5.52 ± 2.564^{b}	2.31 ± 1.208^{b}
SF_1	20.53 ± 2.150^{b}	$44.50\pm0. 927^{b}$	0.33 ± 0.015^{c}	0.25 ± 0.036^{a}	5.26 ± 1.073^{b}	1.72 ± 0.304^{a}
SF_2	15.43 ± 0.307^{a}	41.89 ± 0.709^{a}	0.27 ± 0.013^{b}	0.28 ± 0.011^{a}	4.40 ± 0.212^{a}	1.27 ± 1.103^{a}
SF_3	14.22 ± 1.888^{a}	40.76 ± 0.379^{a}	0.20 ± 0.023^{a}	0.28 ± 0.052^{a}	4.03 ± 0.994^{a}	0.89 ± 0.164^{a}

Superscript letters that differ within the same column denote statistically significant differences, as assessed by one-way ANOVA followed by Tukey's multiple comparison test ($p \leq 0.05$). SM - sample control; SF_1 - 2% fat; SF_2 - 4% fat; SF_3 - 6% fat; *edible membrane (natural) with a diameter of 28-30 mm.

Adhesiveness followed a similar decreasing trend, with significantly lower values in SF_2 and SF_3. This behavior may be attributed to the higher lipid content reducing friction and stickiness, while the membrane itself could have contributed to a more cohesive and smoother surface texture. Springiness and cohesiveness were moderately affected by the treatments. Springiness values decreased significantly from 0.41 in SM to 0.20 in SF_3, indicating a reduction in the elastic behavior of the samples as fat content increased. Cohesiveness, on the other hand, remained statistically unchanged across batches, implying that the structural integrity of the internal matrix was preserved regardless of fat level or membrane presence. Gumminess and chewiness showed the same decreasing trend observed in membrane-free samples, with values significantly lower in SF_2 and SF_3 compared to the control. Chewiness dropped from 2.31 J (SM) to 0.89 J (SF_3), reflecting a

more tender and less resistant texture, likely desirable in terms of sensory attributes. These results confirm that while the edible membrane enhances structural parameters such as hardness, its effect is modulated by the internal composition of the product, particularly fat content. Therefore, optimizing both membrane use and fat level offers a promising strategy to fine-tune the textural properties of heterogeneous meat products while sustainably valorizing by-products.

CONCLUSIONS

The inclusion of beef fat derived from bone by-products, combined with the use of an edible membrane, significantly influences the physicochemical and textural characteristics of reformulated meat products. At moderate inclusion levels (2-4%), fat contributes to a firmer structure and quality improvement by enhancing color intensity and brightness, while higher concentrations (6%) lead to texture softening and reduced elasticity. The edible membrane improves hardness and overall cohesiveness; however, its impact diminishes as fat content increases. Overall, optimizing fat proportion and membrane application can serve as a sustainable and functional strategy for valorizing animal by-products, reducing food waste, and developing products that replace synthetic additives. Upcoming research will aim to conduct an in-depth sensory evaluation of the product and determine its suitability for market introduction.

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FUNCTIONAL EFFECTS OF VEGETABLE BIOINGREDIENTS IN FISH-BASED PRODUCTS: A SYSTEMATIC REVIEW

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Abstract

This systematic review analyzes the scientific literature on the functional effects of vegetable bioingredients in fish products, exploring the mechanisms by which they may influence food quality and health. Studies have highlighted the synergy between vegetable components, such as polyphenols, flavonoids, omega-3 fatty acids and antioxidants, proteins, fatty acids and nutrients in fish, which may lead to improved nutritional profile and reduced risks associated with chronic diseases. The research also suggests that the integration of these vegetable bioingredients into fish products may enhance the bioavailability of nutrients, have antioxidant and anti-inflammatory effects and promote a better balance of intestinal microbiota. The conclusions of this review highlight the potential of functional fish products enriched with plant bioingredients to contribute to the prevention and management of various diseases, providing important support for the development of innovative and sustainable functional foods.

Key words*: fish-based products, sustainable functional foods, vegetable bioingredients.*

INTRODUCTION

Incorporating plant bio-ingredients into fish products can significantly improve their nutritional profile and shelf life. Various studies have shown that mixing fish with plant-based ingredients not only improves the nutritional content but also extends the shelf life of the product. The addition of orange-fleshed sweet potato to fish powder increased the level of beta-carotene from 876.12 to 3182.4 µg/100 g, significantly increasing the vitamin A content (Mekonnen & Aychiluhm, 2024). Using oyster mushrooms and seaweeds such as nori and kombu, which are rich in protein and umami flavor, creates a fish analog that mimics the nutritional profile of fish (Patil et al., 2023). The use of natural additives such as seaweeds and polysaccharides has been shown to reduce lipid oxidation and moisture loss in fish products, thereby extending shelf life (Vijayan et al., 2021; Jannat-Alipour et al., 2019). Fish products enriched with plant ingredients have demonstrated acceptable sensory attributes and maintained their quality over extended periods, with some formulations remaining safe for consumption for up to 90 days (Mekonnen & Aychiluhm, 2024; Jannat-Alipour et al., 2019). In contrast, although plant bioingredients can improve fish products, there may be challenges in terms of consumer acceptance and sensory characteristics, particularly in terms of maintaining the traditional taste and texture of fish. This highlights the need for careful formulation and testing to ensure market viability.

MATERIALS AND METHODS

The systematic review was conducted by consulting the major scientific databases Google Scholar, Web of Science, Scopus and Science Direct from May 2024 to May 2025. The search targeted relevant studies on the effects of plant bioingredients in fish-based food products. The following keyword combinations were used: fish, fish products, fish consumption, nutritional values, plant bioingredients, antioxidant activity, and antimicrobial activity.

Selection process:

1. Elimination of duplicate articles;
2. Evaluation of title and abstract;
3. Full text analysis;
4. Application of inclusion/exclusion criteria;
5. Quality assessment with Risk of Bias Tool.

The inclusion and exclusion criteria used for the selection of studies are presented in Table 1. These criteria were applied to ensure the reviewed literature's relevance, quality, and scientific validity.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Experimental or observational studies on fish products	Studies not on fish-based food products
Articles investigating the effects of plant bioingredients	Papers not assessing clear functional effects of plant bioingredients
Articles published in peer-reviewed journals	Articles without peer-review
Published in English	Published in other languages
Period: 2015-2025	Published before 2015 (with justified exceptions)

The stages of the study selection process, following the PRISMA guidelines, are summarized in Table 2.
Each step of the screening and eligibility assessment is shown along with the corresponding number of articles.

Table 2. Stages of the study selection process according to the PRISMA guidelines - tabular presentation

Stage	Number of articles
Articles identified	100
Duplicates removed	20
Remain after duplication	80
Excluded by title/abstract	21
Excluded after complete analysis	23
Included in the final analysis	36

Risk of bias assessment was performed using the Risk of Bias Tool for randomised controlled trials, taking into account: sequence generation, allocation concealment, blinding of participants/evaluators, data completeness, selectivity of reporting and other sources of systematic error.
The systematic review provides a clear and objective assessment of the available research on the impact of plant bioingredients on fish-based food products, identifying functional and nutritional effects, and will guide future research and industries towards the development of healthier and more sustainable food products.

RESULTS AND DISCUSSIONS

Current trends in fish-based food consumption

Fish is an important component in the daily diet of the population, due to its high content of polyunsaturated fatty acids with essential functional roles in health. With rising incomes, especially in developing countries, fish consumption has been on an upward trend, as it is perceived as a high-quality food (Chen, 2019). Forecasts suggest that by 2050, a significant proportion of the global population will belong to the upper-middle-income class, which will further support the demand for aquatic products associated with important nutritional benefits (Chen, 2019). The urbanization process is also contributing to increased fish consumption through easier access to a greater diversity of food products and increased purchasing power (Sarkodie & Owusu, 2023). Young people in developed countries are showing a growing preference for fish consumption, motivated by health benefits and concerns about the impact of meat consumption (Supartini et al., 2018). In addition, consumer orientation towards more nutritious food choices supports the global expansion of fish consumption (Sarkodie & Owusu, 2023).

Health benefits of consuming fish and fish products

Numerous studies have highlighted the high antioxidant potential of proteins extracted from fish (Gucianu et al., 2023). Chi et al. (2015) and You et al. (2010) reported significant antioxidant activities in protein hydrolysates obtained from the flesh of species such as *Katsuwonus pelamis* and *Misgurnus anguillicaudatus*. Wang et al. (2004) also showed that fish oil supplementation can stimulate the antioxidant enzyme activity of macrophages. Fish is an important source of essential nutrients for human health, with multiple proven benefits such as antioxidant, anti-inflammatory, cardiovascular, liver and neurological protection. Valuable fish components, such as proteins and polyunsaturated fatty acids (PUFAs), help prevent disease and support biological functions by regulating cell signaling pathways

(Chen et al., 2022). More than 40% of global biodiversity is made up of fish and other marine organisms, which are a valuable source of bioactive compounds essential for supporting human health. The bioactive components in fish, including lipids, proteins, vitamins, minerals and various by-products, are recognised for their therapeutic potential. In particular, the beneficial effects of fish consumption are attributed to the high content of long-chain omega-3 polyunsaturated fatty acids (PUFAS), which are easily absorbed by the body. Scientific research shows that, in addition to PUFAs, other bioactive compounds present in fish also contribute significantly to maintaining good health. According to the recommendations of the World Health Organization and the American Heart Association, it is recommended to regularly include fish in the diet (1-2 servings per week) to ensure an intake of 200-500 mg of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), readily available from fatty fish such as salmon or trout (Chiesa et al., 2016; Khalili Tilami et al., 2018; Ashraf et al., 2020).
Table 3 provides a comparative overview of the nutritional composition of three commonly consumed fish species: *Cyprinus carpio*, *Salmo salar*, and *Oncorhynchus mykiss*. The data include energy content, macronutrient distribution, essential minerals, key vitamins, and lipid profiles per 100 g of raw fish. These values, collected from multiple reliable sources, underline the nutritional diversity among species and support the role of fish as a valuable component of a balanced diet.

Table 3. Comparative table of nutritional values (per 100 g raw fish)

Energy values and macronutrients		Species			References
		Cyprinus carpio	*Salmo salar*	*Oncorhynchus mykiss*	
Energy		127 kcal	208 kcal	119 kcal	Karimian-Khosroshahi et al., 2016; Marcu et al., 2010; Vranić et al., 2011; Esaiassen et al., 2022; Rebolé et al., 2015; Skibniewska et al., 2013; USDA, 2023; ANSES, 2020
Proteins		17.8 g	20.4 g	20.1 g	
Carbohydrate		0	0	0	
Minerals	Calcium	19 mg	9 mg	20 mg	
	Iron	1 mg	0.8 mg	0.7 mg	
	Phosphor	210 mg	200 mg	220 mg	
	Magnesium	30 mg	27 mg	25 mg	
	Potasiu	290 mg	370 mg	321 mg	
	Sodium	65 mg	59 mg	51 mg	
Vitamins	Vitamin D	1.5 µg	9 µg	7.5 µg	
	Vitamin B12	2.2 µg	3.2 µg	4.5 µg	
	Vitamin B3	3.5 µg	7.9 µg	5.8 µg	
Fats	Saturated fatty acids	1.3 g	3.1 g	0.9 g	
	Fatty acids Omega 3	0.4-0.8 g	2.6 g	0.9-1.3 g	
	Cholesterol	70 mg	55 mg	59 mg	

EuroFIR AISBL (accessed 01.02.2025).

Valorization of fish in the development of functional food products

The integration of fish products and by-products into various food systems is a promising strategy for the development of functional foods with significant benefits for consumers. This approach contributes to meeting the nutritional requirements of vulnerable population groups, especially in the current context marked by the increased incidence of non-communicable diseases and the challenges posed by global health crises (Pateiro et al., 2021; Gucianu et al., 2024). A relevant example is the valorisation of waste from the processing of rohu fish (*Labeo rohita*), from which protein isolates were extracted and subsequently incorporated into pangasius sausages (*Pangasius pangasius*). The study demonstrated that the addition of the isolates, particularly at a level of 250 g kg^{-1}, led to an improvement in the nutritional value and some physico-chemical properties of the final product without significantly compromising sensory acceptability (Surasani et al., 2022).
Also, the use of fish by-products such as tuna and lobster in the formulation of pasta has

resulted in functional products with superior nutritional profiles. Comparative analysis of these pastes revealed significant differences in fatty acid content, texture and sensory properties. Although pasta with tuna by-product concentrate showed a higher nutritional value, it was found that reducing the tuna content is necessary to optimise sensory characteristics (Ainsa et al., 2021). In addition to by-product valorisation, fish and crustacean-derived enzymes, especially transglutaminases, also offer sustainable alternatives to commercial enzymes used in the food industry. They are characterised by their low temperature activity, thermal lability, high yields and low costs, representing innovative solutions to improve food quality and functionality (Zhang & Simpson, 2020). In addition, a standardised protocol has been developed for the industrial production of fermented fish products based on sturgeon meat (*Acipenser sinensis*), which involves salting, drying, fermentation with *Saccharomyces cerevisiae*, vacuum-packing and sterilisation. The final product obtained is characterised by a favourable sensory profile: rich aroma, reduced undesirable odours, moderate salt content, pleasant texture and attractive colour. Due to the versatility of the method, it applies to both marine and freshwater fish, providing a practical solution for the realisation of long shelf-life fermented snacks (Yang et al., 2019). Last but not least, the addition of cod liver oil, in emulsified or gelled forms, in combination with green tea extract, has demonstrated a positive impact on the technological properties of silver carp (*Hypophthalmichthys molitrix*) sausages. In particular, the gelled emulsion is an effective delivery system of omega-3 fatty acids, contributing to the improvement of the omega-6/omega-3 ratio and the maintenance of product quality during cold storage (Pourashouri et al., 2020). Fish products have a short shelf-life due to their high polyunsaturated fatty acid content and lack of endogenous antioxidants, making them vulnerable to oxidation and microbial contamination, and more effective and natural preservation methods are needed to maintain their quality (Gutiérrez-del-Río et al., 2021). Natural polyphenolic compounds, due to their antioxidant and antimicrobial properties, are a safe and effective alternative to synthetic additives for the prevention of chemical and microbiological deterioration of fish and fish products, helping to maintain their quality during processing and storage (Rathod et al., 2021). The use of natural ingredients with biologically active properties becomes essential to support the functionality of fish-based food products and extend their shelf life. In this context, bio-ingredients of plant origin, recognized for their antioxidant and antimicrobial activity, offer promising solutions from both a technological and nutritional point of view.

The role of plant bioingredients in the sensory, oxidative and microbiological protection of functional fish-based foods

The integration of fish bioactive compounds in the formulation of functional food products is a promising research direction aiming at optimizing the nutritional value of modern diets. Effective preservation strategies, preferably based on natural antioxidants and antimicrobial ingredients, need to be used to ensure the stability of these products and to prolong their shelf life, and can combat contaminants that may come from animal feed (Ciobanu et al., 2024; Matei et al., 2024; Anchidin et al., 2023). In this regard, bioingredients of plant origin have gained increased interest due to their efficiency in inhibiting oxidative and microbial processes, without compromising food safety or sensory acceptability from the consumers' perspective (Ciobanu et al., 2024; Ciobanu et al., 2025; Manoliu et al., 2024). Combining valuable animal resources, such as bioactive fish compounds, with functional plant extracts thus provides an integrated framework for the development of sustainable, safe and innovative food products. Perceptions of the healthiness of foods play a key role in product choice, with health-motivated consumers more likely to seek information or pay attention to cues that allow them to assess the nutritional benefits of available food options (Mitterer-Daltoé et al., 2014; Ciobanu et al., 2024). Phytocompounds, in particular those of the polyphenol class, have a high potential in technological applications related to fish products, contributing to their oxidative

stabilisation and shelf-life extension. Citrus peels, due to their high content of phenolic acids and flavonoids, are recognised for their remarkable antioxidant activity, often superior to synthetic antioxidants. Similarly, the use of extracts from cabbage leaves (*Brassica oleracea* var. *capitata* L.) and banana peels (*Musa* sp. L.) in fish-based food formulations has shown a significant improvement in oxidative stability. Phenolic antioxidants from these plant sources neutralise free radicals resulting from lipid oxidation, thus helping to reduce degradation processes and maintain the sensory characteristics of the final product (Gutiérrez-del-Río et al., 2021).

Ali et al. (2019) used a Box-Behnken experimental design to optimize the extraction parameters to maximize the yield of total phenolic compounds (TPCs) from plant by-products such as cabbage leaves and banana peels. The obtained extracts were chemically characterised and subsequently incorporated at concentrations of 0.5%, 1% and 1.5% into fish meat products to evaluate the influence on lipid oxidative stability and sensory properties during storage at refrigerated (9 days) and frozen (60 days) temperatures. In the context of current food safety concerns, recent research is increasingly focusing on the use of natural preservatives as an alternative to synthetic additives. By-products resulting from vegetable processing are promising sources of natural antioxidants, the use of which in the food industry can generate economic benefits for producers and improve the nutritional value of consumer products (Boișteanu et al., 2025).

Figure 1 illustrates the main natural antioxidant compounds evaluated in fish-based food products, along with their reported effects. This graphical representation, adapted from Gutiérrez-del-Río et al. (2021), highlights the functional roles of various plant-derived bioactive compounds in enhancing the oxidative stability and overall quality of fish products.

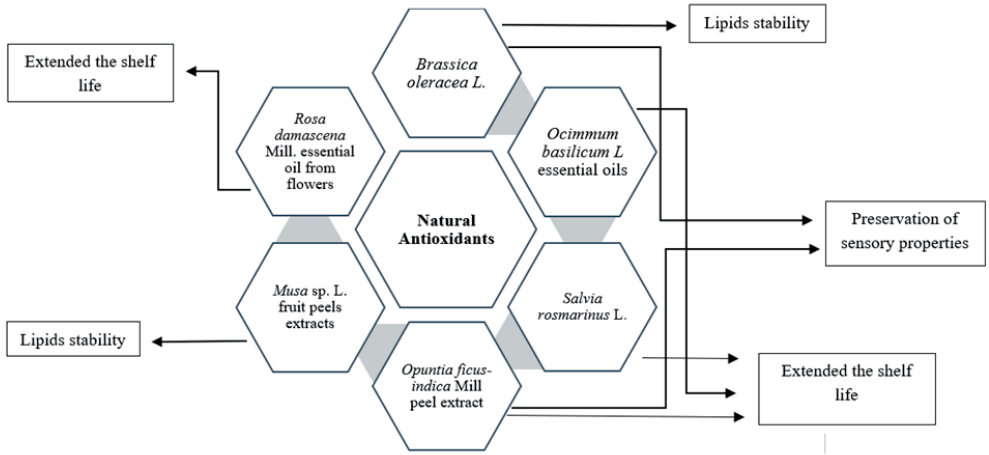


Figure 1. Graphical representation of the main natural antioxidant compounds tested in fish food products and their effects according to Gutiérrez-del-Río et al. (2021)

Plant bioactive compounds, such as polyphenols, flavonoids and tannins, exert a protective role in the food matrix of fish products, in particular through mechanisms that inhibit lipid and protein oxidation. At the molecular level, these compounds act as electron or hydrogen atom donors, neutralising free radicals (such as the peroxyl radical ROO- or hydroxyl radical OH-), which are responsible for the initiation and propagation of unsaturated fatty acid oxidation chains in fish. Polyphenols bind to free radicals formed during oxidative stress and form stable species, stopping the chain reactions of lipid peroxidation. Flavonoids can chelate transition metal ions (Fe^{2+}, Cu^{2+}), which catalyse the Fenton and Haber-Weiss reactions responsible

for the generation of highly reactive hydroxyl radicals. Tannins form complexes with proteins and protect them from oxidative degradation, thus maintaining the texture and nutritional value of the product (Deng et al., 2025; Lipşa et al., 2024).

To highlight the functional potential of various plant-based bioingredients in fish-based products, several studies have investigated their antioxidant and antimicrobial properties. Table 4 below summarizes key findings regarding the concentrations used, types of fish products tested, and the observed preservation effects. These natural extracts, often applied in small percentages, have shown promising results in enhancing shelf life and maintaining product quality, making them suitable candidates for clean-label reformulations.

Table 4. Functional effects of vegetable bioingredients used in fish-based products

Vegetable bioingredient	Concentration %	Fish food product	Observed effect	Ref.
Salvia rosamarinus extract	0.02	Fish Patties	Delayed lipid oxidation reduced microbiological spoilage during cold storage.	Martínez et al., 2019
Punica granatum extract	0.02	Fish Patties	Significant antioxidant and antimicrobial activity, contributing to extended shelf life.	Martínez et al., 2019
Olea europaea extract (hydroxytyrosol)	0.02	Fish Patties	Reduced lipid oxidation and microbiological spoilage, extending product shelf life.	Martínez et al., 2019
Nymphaea nouchali extract	10.0(w/v)*	Frozen Tilapia Fillets	Shelf-life extension through inhibition of lipid oxidation, microbial growth, and maintenance of sensory quality.	Dulal et al., 2023
Sambucus nigra extract	0.005-0.01	Salmon Burgers	Antioxidant and antimicrobial effects help maintain product quality during storage.	Jonušaite et al., 2021

*Extract types: water (0E), 50%, 75%, and 100% ethanol

In addition to these antioxidant functions, many phenolic compounds also possess antimicrobial properties, affecting the membrane permeability of bacteria or interfering with their metabolism, contributing to shelf-life extension. Phenolic compounds (e.g. flavonoids, phenolic acids) can interact with bacterial membrane lipids, leading to disruption of the lipid bilayer structure, causing loss of membrane integrity. Increased membrane permeability allows essential ions (K^+, H^+) and other metabolites to escape. The decrease in membrane potential, affects the functioning of ion pumps and active transport, and in Gram-negative bacteria, the compounds can destabilize the lipopolysaccharide layer (LPS). This leads to a loss of protons and ATP, affecting the energy of the bacterial cell. Some phenolic compounds act on essential bacterial enzymes, may inhibit enzymes involved in glycolysis, the Krebs cycle or the respiratory chain, and bind sulfhydryl (-SH) groups of proteins or interfere with metal groups (Fe, Cu) in redox enzymes. They act as pro-oxidising agents in the bacterial cell, generating reactive oxygen species (ROS) that cause oxidative stress. Phenolic compounds can interfere with DNA, blocking replication or transcription, inhibit ribosome activity, affecting protein synthesis, and in some cases, phenols can form chelates with metal ions required for replication processes or DNA stability. Some phenolic compounds induce an internal oxidative stress in the bacterial cell through redox imbalance. This may activate the apoptotic cascade in some sensitive bacteria. All these mechanisms lead to the cleavage of microorganisms, stopping bacterial multiplication and slowing down the spoilage of food products by prolonging shelf life (Lipşa et al., 2024; McGurrin et al., 2025).

CONCLUSIONS

The findings of this systematic review highlight the significant potential of functional fish products enriched with plant bio-ingredients in the context of current consumer trends towards healthy and sustainable food. The regular consumption of fish and fish products is well documented for its health benefits due to its omega-3 fatty acids, high-quality protein and essential micronutrients. The integration of plant bioingredients in these products not only capitalizes on fish resources through the development of innovative functional foods but

also contributes to protection against oxidation and microbiological contamination, thus improving food safety and sustainability. The synergy between bioactive plant compounds (such as polyphenols and flavonoids) and fish-specific compounds enhances health benefits by promoting nutrient bioavailability, gut microbiota balance and reducing the risk of chronic diseases. These results support the development of advanced functional food products with high nutritional value and a positive impact on public health.

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FORMULATION OF A FUNCTIONAL MEAT PRODUCT WITH A COMPACT STRUCTURE BY INCORPORATING *Cetraria islandica*, A UNDEREXPLOITED INGREDIENT, AND EVALUATION OF ITS POTENTIAL IN THE MEAT INDUSTRY

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Abstract

Nowadays, consumer demands for more natural products have forced the food industry to increasingly exclude synthetic compounds from its products and to explore natural sources of bioactive ingredients such as natural antioxidants. All parts of plants, such as fruits, nuts, seeds, leaves, roots and barks, contain antioxidant compounds, making them potentially valuable ingredients for the food industry in the production of products with functional properties. In order to improve the functional properties of meat products, we have developed a meat product assortment with a compact structure, incorporating lichen (Cetraria islandica), an antioxidant ingredient that is unconventional for this type of products, thus exploring new ways of innovation in the meat industry. In order to compare the effect of the addition of Cetraria islandica, we manufactured three batches (1%, 3%, 5%) and a control batch. The resulting products were analyzed internally and externally for physicochemical quality and antioxidant capacity, with highly significant differences ($p<0.001$) observed between the internals and externals, and sensory evaluations were performed on the whole product.

Key words*: antioxidants, functional food, meat products, sensory analysis, quality analysis.*

INTRODUCTION

Since ancient times, meat has been a staple of the human diet, as it is a nutrient-rich food high in protein, vital amino acids, vitamins (especially B vitamins) and minerals such as iron and zinc (Bis-Souza et al., 2019; Manoliu et al., 2024; Boișteanu et al., 2023; Ianițchi et al., 2024) that fulfill various functions in the human body (Manoliu et al., 2023) and that can be more easily assimilated from meat than from other foods (Ciobanu et al., 2024). These include maintenance of normal physiological processes (Anchidin et al., 2024a), increased immunity and avoidance of certain diseases such as malnutrition (Ciobanu et al., 2023). However, consumers perceive meat consumption as linked to various health hazards due to high fat, salt and additives content.

One of the most important industries in the world is the meat sector (Boișteanu et al., 2025). The demand for meat and meat products has increased in different regions of the world over the last 20 years (Anchidin et al., 2024b), tripling globally in the last 50 years (Ciobanu et al., 2025). Currently, one of the main forces behind new trends and breakthroughs in the creation of new healthy products, including those derived from meat, is the growing market demand for healthier and more functional foods (Ruiz-Capillas & Herrero, 2021). This trend also relates to meat products, as consumer predilection for animal products is likely to persist. (Kumar et al., 2013). By reducing consumption of foods that are commonly consumed, functional foods aim to reduce the prevalence of chronic diseases. Large food corporations, including the meat industry, are becoming very interested in formulating foods based on the potential benefits their non-nutritional constituents may have for the consumer (Fernández-Ginés et al., 2005).

Functional foods are a remarkable and interesting category of foods with health-promoting qualities, such as anti-carcinogenic, antioxidant and cholesterol-lowering qualities, which make them attractive to consumers (Anchidin et al., 2023).

The need to provide enhanced food products with components that promote health and prevent nutrition-related diseases is well recognized in the industry (Mironeasa & Ungureanu-Iuga, 2024), as consumers are becoming increasingly demanding about the type of food they buy and consume (Pogurschi et al., 2018; Ciobotaru et al., 2024a). In response to increasing consumer demands, the food industry is reformulating food products to enhance the physiological performance of natural nutrients or by incorporating a bioactive component (Anchidin et al., 2023; Ciobotaru et al., 2024b). One way to procure improved/ functional foods in modern food processing involves the use of different food enhancers, such as plant agents (Zugravu et al., 2017). Studies on potential applications of different products and by-products from plants, lichens and algae in the food sector, as well as studies to better understand these concerns, have been conducted in increasing numbers. The aim of these studies is to capitalize on non-traditional sources of bioactive chemicals to sustainably improve the nutritional content of staple foods or meat quality (Mironeasa & Ungureanu-Iuga, 2024).

In nature, lichen is a unique type of plant (Calcott et al., 2018). It is a mutualistic entity consisting of a fungus (mycobiont) and an alga (photobiont) allied with a robust and specific composition (cyanobacteria or other microbial species) (Thakur et al., 2023). Some metabolites and minerals found in lichens are a valuable source of nutritious food for invertebrates and vertebrates (humans and some reptiles), mites, termites, snails, caterpillars, etc. *Cetraria Islandica*, sometimes known as Icelandic moss, has been consumed as food in a number of nations including Scandinavian nations, Sweden, Norway and Iceland and Bosnia and North Africa mainly (Sharma & Mohammad, 2020).

Because of the bioactive substances they contain, lichens are extremely important for nutrition. Long used as a food, lichen is a multifunctional, nutrient-rich food that can be consumed almost every day. These invaluable natural resources are now being exploited for a variety of other applications (Thakur et al., 2023).

Lichens are notable for their production of unique primary and secondary metabolites, including amino acids, polysaccharides (lichenin, isolichenin), vitamins and pigments. Lichen polysaccharides, mainly composed of galactomannans, α-glucan and β-glucan, exhibit diverse biological properties. According to recent research, the metabolites generated by lichens have substantial biological potential and active properties such as antifungal, antibacterial, anticarcinogenic, antiviral, antioxidant and anti-inflammatory properties (Bao & Bau, 2013; Huang et al., 2018; Olafsdottir & Ingólfsdottir, 2001; Ullah et al., 2019). Among the biologically active components that *Cetraria islandica* contains, phenolic compounds, diterpenoids, depsides, and various benzenoid and non-benzenoid derivatives have been identified (Yusuf, 2020).

Because they contain more than 500 potentially bioactive substances that have been discovered to date, lichens (phylum *Lichenophyta*, to which *Cetraria islandica* belongs), the least-utilized subdivision of fungi, are composite plants used in folk medicine to treat a variety of ailments, from digestive to respiratory. The chemical substances they contain, which include lichenic acids such as usnic acid, lobar acid, lecanoric acid or salazine acid, are cytotoxic, antioxidant and antibacterial agents (Patriche et al., 2019).

There are two types of antioxidants: natural and synthetic (Flocea et al., 2024). When developing new meat products, it is important to keep in mind that chemically and biologically synthesized products can block food poisoning. The food industry utilizes a variety of natural and synthetic materials that are gatekeepers against oxidative and microbiological spoilage to stop these events (Krotova et al., 2023). However, a significant element influencing the acceptability of food and food innovation products is their perceived naturalness (Siegrist & Sütterlin, 2017). Thus, the use of natural antioxidant ingredients represents a promising alternative for the meat industry.

According to research conducted by Gülçin et al. (2002), in comparison with various standards including α-tocopherol, BHA, BHT and quercetin, *C. islandica* exhibits strong antioxidant activity, reducing power, DPPH

radical and superoxide anion scavenging capacities. The study findings indicate that *C. islandica* may find successful applications in the pharmaceutical or food/food supplement industry.

Due to the information collected so far from the literature on *Cetraria Islandica* and as a way to innovate in the meat industry by using sources with extremely restricted utilization we decided to develop three batches of pastrami-type products with added lichen. This, in our opinion, is an area with potential but limited research. In order to explore the benefits of *Cetraria islandica*, we aimed to analyze the effects of its addition in different concentrations on the quality and bioactivity of meat products.

MATERIALS AND METHODS

In the framework of this research, four batches of pastrami (made out of pork leg) were manufactured, three of them with 1, 3 and 5% added *Cetraria islandica* lichen in their composition, and the fourth batch was the control batch (without added *Cetraria islandica*) in order to perform a comparative qualitative analysis between them. All the described batches were manufactured at the "Ion Ionescu de la Brad" Iasi University of Life Sciences (IULS). The raw material, consisting of boneless pork leg, was purchased from SC SAGROD SRL (Botoșani, Romania), the lichens *Cetraria islandica* were purchased from SC STEFMAR PRODUCȚIE SRL (Râmnicu Vâlcea, Romania) and the seasoning ingredients used were purchased from a company specialized in their commercialization in Iasi County, SC Rocas FDS SRL (Iasi, Romania).

The experimental batches, in addition to the meat used and the addition of *Cetraria islandica*, also contained the following spices: salt, ground black pepper, garlic powder and sweet paprika. The lichen (*Cetraria islandica*) powder was solubilized in a 10% brine solution and subsequently introduced into the meat pieces by vacuum tumbling, allowing the penetration of the solution into the muscle tissue.

The spices were integrated in a batter through which the meat pieces were passed, followed by hanging them on the rack trolley and putting them through the heat treatment, the stages of which are shown in Table 1. After completion of this, the samples were cooled, vacuum-packaged and stored at refrigeration temperatures (0-4ºC) until physico-chemical and phytochemical analyses were carried out.

The physico-chemical analyses consisted in the analysis of the pH of the samples, colorimetric parameters, textural profile, protein content, fat content, moisture content, dry matter, ash, collagen and salt content. The phytochemical content of the samples and their antioxidant capacity were also analyzed.

The pH values of the samples were carried out with pH meter HI98163 with electrode FC23232323 (SC Hanna Instruments SRL).

The color analyses of the samples, which involved the analysis of color parameters in three-dimensional CIELAB space, were performed using the Konica Minolta CR-410 colorimeter (Konica Minolta, Inc.).

Table 1. Heat treatment applied to the experimental batches

Experimental batches	Heat treatment							
	Drying I		Smoking		Boiling		Drying II	
	T (ºC)	Time (min)	T (ºC)	Time (min)	T (ºC)	Time (min)	T (ºC)	Time (min)
P0L P1L P3L P5L	60	30	65	40	75	-	80	20

P0L - control pork leg (no added lichens); P1L - pork leg with 1% added lichens; P3L - pork leg with 3% added lichens; P5L - pork leg with 5% added lichens.

The colorimetric parameters Hue (h°), Chroma (C*), and visually perceived color difference between samples (ΔE) were also calculated using formulas (1), (2), and (3) based on the data collected from these parameters. Hue (hº) and Chroma (C*) were computed following the approach described by Salueña et al. (2019), while ΔE was calculated according to the

method proposed by Wei et al. (2012). The textural profile of the samples (TPA) was carried out with the TA1+1K Plus textuometer (Ametek, Inc.), equipped with a cylindrical compression probe, which performed a double compression. The following parameters were measured during this analysis: hardness, cohesiveness, elasticity, adhesiveness, gumminess, chewiness.

$$H° = \arctan \frac{b^*}{a^*} \quad (1)$$

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (2)$$

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (3)$$

The chemical, colorimetric and phytochemical analyses performed on the analyzed samples were carried out both at ~0.3 cm from the surface of the products and inside the products, as differences in the absorption of the brine solution with lichens inside the samples were noticed. This is due to the increased viscosity of the solution caused by the addition of lichens which made it difficult for the solution to penetrate deep into the meat pieces.

Proximate composition analysis of moisture, protein, collagen, fat, ash and salt content were carried out using the Food Check Meat Analyzer (Bruins Instruments GmbH, KPM Analytics), a near-infrared spectroscopy (NIR) based system, recognized for its flexibility and accuracy in quantitative analysis of meat composition.

To evaluate the antioxidant capacity of the analyzed samples, the first step was the extraction of bioactive compounds. For this purpose, 1 g of sample was taken from *Cetraria islandica* powder and meat samples and mixed with 10 ml of 70% ethanol as an extractive solvent. The preparation of the extract for the determination of flavonoids and polyphenols followed the first method described by Trifunschi et al. (2017), only using ethanol instead of methanol. The determination of antioxidant capacity by DPPH method was performed according to the method described by Pires et al. (2017), and the ABTS method according to the method of Dumitrașcu et al. (2022).

The Specord Plus 210 UV-vis spectrophotometer Specord Plus 210 UV-vis (Analytik Jena, Germany) was used to measure the absorbance of the materials. Heating in an ultrasonic water bath and, in some cases, centrifugation was followed by sampling to ensure reproducibility. Flavonoids were expressed in mg/g DW, polyphenols in mg GAE/g DW, DPPH assay results in µMol Trolox/g DW and percent (inhibition), and ABTS assay results in µMol Trolox/g DW and percent (inhibition). The solutions used for the analyses of phytochemical content and antioxidant capacity were purchased from Sigma Aldrich Steinheim (Darmstadt, Germany) for antioxidant assays.

In addition to the objective analysis presented above, the sensory evaluation of the samples was carried out as a subjective method to assess the quality of the experimental batches realized in this study. The analysis was performed by a panel of 12 semi-trained tasters (9 women and 3 men). The decision to include this stage was motivated by the importance of consumer acceptability as an imperative factor in the success of functional food products. Sensory evaluation involves the analysis of consumer reactions based on perceptions generated by the human senses (visual, olfactory, gustatory, tactile and auditory), having a major influence on the perception of overall product quality (Ciobanu et al., 2023). The analysis was performed 72 hours after the preparation of the experimental batches under blind test conditions, the samples were coded with randomized three-digit numerical combinations. In this study, we used the external PrefMap analysis, an advanced sensory method based on multivariate data, which allows the correlation between quantitative scores of sensory attributes and preferences expressed by consumers (Boișteanu et al., 2025).

All parameters were analyzed in ten replicates per sample, with the exception of the bioactive compound determinations, which were carried out in five replicates.

RESULTS AND DISCUSSIONS

Table 2 presents the results of the chemical composition of the analyzed samples (mean ± SD) which are composed of: fat, dry matter, humidity, protein, collagen, salt and pH (this represents the only physical parameter in this table). The analyses were performed by taking samples from ~0.3 cm from the surface of the meat pieces and from their central area in order to highlight possible compositional differences, as presented in the Table 2. For the control batch P0L, differentiated analyses were not performed for the subsurface and interior, because this sample does not contain lichens that would limit the distribution of the brine solution in the center of the sample and create qualitative differences.

Statistical differences identified as significant ($p < 0.05$) between batches and between the analyzed regions are highlighted by lowercase letters (for the subsurface area) and uppercase letters (for the interior area).

The addition of lichens influenced the fat content of the samples in both analyzed regions, but especially in the samples taken from the subsurface where more pronounced differences are observed between the P0L sample (control sample) and the samples with 1, 3 and 5% addition of lichens. As for the samples taken from the inside, no significant differences ($p > 0.05$) are observed between the P0L sample and the sample with 1% addition of lichens (P1L). Very significant differences ($p < 0.05$) are observed with the addition of 5% *Cetraria islandica* in terms of the decrease in the fat content inside the samples compared to the P0L batch. It can be seen that with the increase in the concentration of *Cetraria islandica*, a decrease in the fat content occurs in both analyzed regions. Even though the values obtained in the samples taken from the central area of the samples do not present such obvious differences as those taken from the subsurface area, an influence caused by the addition is still observed within the samples.

The statistical interaction between interior and surface for fat shows very significant differences ($p < 0.001$) for all 3 batches with the addition of *Cetraria islandica*, from which we can deduce that it is not completely absorbed to the center of the samples, but it is still absorbed as shown by the results recorded in Table 2.

The moisture content of the samples and their dry matter content were also influenced by the addition of *Cetraria islandica*. The samples taken from the subsurface region for both parameters did not show significant differences ($p > 0.05$) between the analyzed samples, which shows that this addition helps to better retain the moisture of the samples, especially in the external area, where most often there are higher moisture losses compared to the internal area. Also, an initial decrease in the moisture content of the samples and an increase in the dry matter in the sample with 1% addition (P1L) compared to the control sample (P0L), from 70.81 ± 0.26% to 70.70 ± 0.00% and, respectively, 29.19 ± 0.26% to 29.30 ± 0.00%. In the sample with 3% addition (P3L), equal values are observed for the two mentioned parameters, and as for the P5L sample, a slight increase in humidity (71.16 ± 0.26%) and a decrease in dry matter (28.85 ± 0.26%) is observed in the samples in the subsurface area, which indicates that the addition of *Cetraria islandica* helps to retain water in the samples, especially in the area where the meat is more prone to moisture loss and to reduce losses following heat treatment, even if the differences in the subsurface area for the dry matter and humidity parameters do not differ significantly between the samples ($p > 0.05$). As for the analyses of the samples from the central area of the meat pieces, they present significant differences ($p < 0.05$) between the P0L control sample and the other samples studied. At present, we have not identified any articles indicating an influence of the addition of *Cetraria islandica* on the moisture or moisture retention capacity of meat samples, but our results indicate such an influence. However, the study by Antropova et al. (2021) shows that lichens contain a high amount of hydrophilic polysaccharides that can contribute to increased water retention in meat products. The statistical differences between the samples analyzed at the subsurface and those from the interior are highly significant for all samples with studied additions ($p < 0.001$), as can be seen in Table 2.

The mean values of protein and collagen parameters show initial increases in the P1L

sample compared to the P0L sample for samples taken from the subsurface region, followed by a successive decrease in the P3L and P5L samples. The samples taken from the center of the meat pieces show a decrease in the values for protein and collagen in the P1L samples (20.50 ± 0.07% and, respectively, 18.56 ± 0.15%) and P3L (19.84 ± 0.12% and, respectively, 18.15 ± 0.15%) compared to the control sample, with significant differences observed between them ($p < 0.05$). However, in the P5L sample, a decrease is no longer observed, but a maintenance of the values of these parameters identical to those present in the P3L sample. These results indicate a common trend, both in the superficial and central layers, of decreasing protein and collagen losses with increasing *Cetraria islandica* concentration, especially at the 5% concentration. However, the statistical differences between the internal and superficial areas were highly significant for protein and collagen parameters ($p < 0.001$) in most of the analyzed batches. A notable exception was batch P5L, where the mean collagen content values did not show significant differences between the two sampling regions ($p > 0.05$), suggesting a uniform distribution of collagen in the product at the maximum lichen concentration used.

The salt content of the samples shows an initial increase in the value for the subsurface region from 1.71 ± 0.03% in the control sample to 1.81 ± 0.11% in the sample with 1% lichen addition (P1L), the differences between them being significant ($p < 0.05$). In the samples with 3% (P3L) and 5% (P5L) addition within the same analyzed region, a constant decrease in salt content is observed with the increase in lichen content below the value of the control sample and inherently below the value of the P1L sample, obtaining values of 1.66 ± 0.05% (P3L) and 1.58 ± 0.07 (P5L), which show significant differences ($p < 0.05$) compared to the P1L and P0L samples. These values are correlated with the increase in moisture content in the samples with 3 and 5% lichen, which indicates a dilution of the salt content and also the ability of the lichen to reduce the salt content of the meat samples, results that are in accordance with those presented by Thakur et al., 2023 which demonstrate the ability of lichens to reduce salt in food, thus having beneficial effects on health by reducing the risk of hypertension. The salt content shows an even more pronounced decrease in the samples from the central region of the samples, with extremely significant differences ($p < 0.001$) in the case of the P1L sample and very significant ($p < 0.01$) in the case of the P5L sample. These differences mainly indicate a limitation of the penetration of the salt solution inside the samples caused by the viscosity of the solutions with the addition of lichen. However, the P3L sample does not show significant differences ($p > 0.05$) between the interior and subsurface regions.

The pH values were also highly significantly different between the two regions ($p < 0.001$), with lower values in the superficial layer. The pH values of the sample with the addition of 1% lichen (P1L) showed a slight increase in both the subsurface region (5.92 ± 0.017%) and the interior region (6.08 ± 0.02%) compared to that of the control sample P0L (5.90 ± 0.05%), with significant differences observed between it and the interior P1L sample ($p < 0.05$), but insignificant in the subsurface region of the same sample ($p > 0.05$). The P5L sample achieved the greatest reduction in pH value in the subsurface region, being 5.81 ± 0.03%, the differences between it and the other samples taken from the same region (subsurface) being significant ($p < 0.05$). The P3L sample for the same region recorded an intermediate value between P5L and P1L, of 5.89 ± 0.02%, which does not show significant differences compared to the P0L and P1L samples ($p > 0.05$), but only compared to the P5L sample ($p < 0.05$). The more pronounced decrease in pH caused by the increase in the *Cetraria islandica* content is most likely due to its 3-5% content of organic acids (usnic, protolichesterinic, protocetraric, and others) which have a biological and biochemical role, as shown by the research of Podterob (2008).

Regarding the pH of the samples from the central region, it recorded higher values than in the subsurface region, which indicates a weaker acidic character. The sample from the central region with the highest amount of lichen, P5L, presented the lowest pH value, of 5.93 ± 0.05% compared to the other samples taken from the same region, the differences between it and the

control sample (P0L) being not significant ($p > 0.05$). Regarding the statistical correlations with the P3L and P5L samples, the P1L and P0L samples present significant differences ($p < 0.05$). Also, the statistical differences between the 2 studied regions (subsurface*center) for all the analyzed samples present extremely significant differences ($p < 0.001$) between them (Table 2). Table 3 presents the values of the colorimetric parameters L*, a*, b*, hue (h°), chroma (C*) and the total color difference (ΔE) between the brown batch and the batches with the addition of 1%, 3% and 5% *Cetraria islandica*, determined both at ~0.3 cm from the surface of the samples (subsurface) and inside them. Small and large letters indicate statistically significant differences ($p < 0.05$) within each row, for the superficial area and the internal area, respectively.

Table 2. Chemical composition of the studied batches (mean ± SD; n = 10)

Studied chemical parameters		Batches			
		P0L	P1L	P3L	P5L
Fat	Subsurface	$6.78 \pm 0.37^{c,B}$	5.84 ± 0.15^{b}	5.75 ± 0.22^{b}	5.31 ± 0.28^{a}
	Center		6.51 ± 0.10^{B}	6.41 ± 0.47^{AB}	6.11 ± 0.25^{A}
Dry Matter	Subsurface	$29.19 \pm 0.26^{a,C}$	29.30 ± 0.00^{a}	29.19 ± 0.75^{a}	28.85 ± 0.26^{a}
	Center		28.56 ± 0.20^{B}	28.14 ± 0.45^{A}	27.99 ± 0.29^{A}
Humidity	Subsurface	$70.81 \pm 0.26^{a,A}$	70.70 ± 0.00^{a}	70.81 ± 0.75^{a}	71.16 ± 0.26^{a}
	Center		71.44 ± 0.20^{B}	71.86 ± 0.45^{C}	72.01 ± 0.29^{C}
Protein	Subsurface	$20,69 \pm 0.13^{a,C}$	21.26 ± 0.14^{b}	20.80 ± 0.40^{a}	20.76 ± 0.28^{a}
	Center		20.50 ± 0.07^{B}	19.84 ± 0.12^{A}	19.84 ± 0.12^{A}
Collagen	Subsurface	$18,89 \pm 0.11^{c,C}$	19.11 ± 0.14^{d}	18.52 ± 0.15^{b}	18.25 ± 0.22^{a}
	Center		18.56 ± 0.15^{B}	18.15 ± 0.15^{A}	18.15 ± 0.14^{A}
Salt	Subsurface	$1.71 \pm 0.03^{b,C}$	1.81 ± 0.11^{c}	1.66 ± 0.05^{ab}	1.58 ± 0.07^{a}
	Center		1.52 ± 0.10^{AB}	1.60 ± 0.00^{B}	1.45 ± 0.11^{A}
pH	Subsurface	$5.90 \pm 0.05^{b,A}$	5.92 ± 0.017^{b}	5.89 ± 0.02^{b}	5.81 ± 0.03^{a}
	Center		6.08 ± 0.02^{C}	5.98 ± 0.04^{B}	5.93 ± 0.05^{A}
Statistical significance (p-value) between Subsurface*Center	Fat	-	***	***	***
	Dry Matter	-	***	***	***
	Humidity	-	***	***	***
	Protein	-	***	***	***
	Collagen	-	***	***	ns
	Salt	-	***	ns	**
	pH	-	***	***	***

P0L - control pork leg (no added lichens); P1L - pork leg with 1% added lichens; P3L - pork leg with 3% added lichens; P5L - pork leg with 5% added lichens.
Values within the same row followed by different lowercase letters (a, b, c, d) differ significantly ($p < 0.05$) for subsurface samples; Values followed by different uppercase letters (A, B, C, D, E) differ significantly ($p < 0.05$) for interior samples.
Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The L* values in the subsurface area registered an initial decrease in the batch with 1% addition of *Cetraria islandica* (63.69 ± 0.67%) compared to the control batch (64.97 ± 0.89%), the differences between them are significant ($p < 0.05$). In batches P3L and P5L in the subsurface area, the mean values of L* increased progressively with the addition of lichen, indicating an intensification of the product's brightness, up to the maximum value of 73.73 ± 0.50%, observed in batch P5L. The differences between all batches analyzed for the colorimetric parameter L* analyzed in their subsurface region were significant ($p < 0.05$). The same trend was also observed in the interior region of the samples for this parameter (L*), except that within it and the P1L sample increased compared to the control sample. As with the samples from the subsurface region, the analyses performed in this region also showed significant differences between all the samples studied ($p < 0.05$). Also, the statistical differences between the analyzed regions showed highly significant differences for all the analyzed lots ($p < 0.001$).

The results for the colorimetric parameter L* can be correlated with the results for the average humidity of the analyzed samples (Table 2). The increase in the average humidity value is directly proportional to the increase in

the brightness values of the samples. Also, the P1L (subsurface area) sample is the only one that recorded an average humidity value lower than the control sample (Table 2), the same situation being identified in the case of the L* parameter (Table 3).

The colorimetric parameter a*, which indicates the intensity of the red-green colors (red for positive values and green for negative values) of the samples, decreased successively with the increase in the concentration of *Cetraria islandica*, both on the surface and inside, the decrease being more pronounced in the case of the samples analyzed from the Subsurface region. Lot P5L recorded the lowest average value (3.19 ± 0.43 within the subsurface region), which indicates a slight change in the specific color of the meat towards the color of the lichen. The more pronounced decreases of the a* parameter in the area closer to the outside of the samples show a limited penetration of the brine solution with lichens inside the meat samples due to its viscosity.

Regarding the statistical differences between the mean values of the samples from the subsurface region and the interior region, significant differences were recorded in the case of the P1L group ($p < 0.05$), insignificant in the case of the P3L group ($p > 0.05$) and extremely significant in the case of the P5L group ($p < 0.001$).

The colorimetric parameter b* increased significantly with the addition of lichen, from the minimum recorded in the P0L control sample (9.01 ± 0.37) to the maximum in the P5L batch (11.92 ± 0.42, subsurface region). This result suggests that *Cetraria islandica* induces a slight intensification of the yellow shade in the final color of the products. The interior area of the samples, where the lichen brine solution does not have a very good penetration, presents higher values of the b* parameter, which may indicate that it is not necessarily the addition of *Cetraria islandica* that leads to the increase of this parameter, but the increase in the humidity of the samples (Table 2) which, most likely, causes the decrease of hemin pigments. For this parameter, the statistical differences between the subsurface and the interior are very significant for the P1L and P3L samples ($p < 0.01$) and extremely significant in the case of the P5L sample ($p < 0.001$).

The hue angle (hº) followed a clear downward trend. This change is especially evident in the P5L group, which presents the lowest values (subsurface - 12.75º, interior - 26.67º). The high values of hue correspond to a visually less "yellow" and more "reddish" tone, which is not consistent with the decrease in the colorimetric parameter a* and may be caused by the presence of negative values in the data set for the a* parameter that caused the direct relationship between the hue angle values, calculated based on the calculation relationship, to no longer be easily correlated, as shown by McLellan et al. (1995). The lower values and in the case of some negative samples of the a* parameter and the positive values of the b* parameter suggest a shift of the chromatic vector in quadrant II of the a*-b* plane, specific to the Hunter Lab space (McLellan et al., 1995), which corresponds to a yellow-green dominance. The statistical differences between the P0L control sample and the samples with the addition of lichens were significant ($p < 0.05$). The statistical interaction between the two analyzed regions of the same sample presented extremely significant statistical differences only within the P5L group ($p < 0.001$), in the case of the other groups no significant differences were identified ($p > 0.05$).

The perceived color intensity, chroma (C*) showed reductions in its value in the lichen-added and subsurface groups, with the lowest level identified in the P5L group (12.34 ± 0.43). The statistical differences between the values in the subsurface group do not show statistical differences ($p > 0.05$) for the lichen-added groups. The statistical differences are found only between these groups and the control group, which are significant ($p < 0.05$). These results show that the addition of lichens influences the perceived color saturation of the samples. In contrast, the samples in the interior region with lichen addition show a very slight decrease in color saturation (C*) for the P1L and P3L samples, which does not represent a significant difference ($p > 0.05$). However, there are significant differences between the P5L sample and the P1L and P3L samples ($p <$

0.05), but insignificant compared to the P0L control sample ($p > 0.05$).

The visually perceived color difference (ΔE) was significantly different for all samples analyzed within the same region ($p < 0.05$) (Table 3), especially within sample P5L, where ΔE reached mean values of 11.85 ± 0.97 (subsurface) and 8.41 ± 1.08 (interior). These values indicate visual changes clearly perceptible to the naked eye, confirming that the addition of lichen strongly influences the visual appearance of the products that are the subject of this research. The differences between regions in the case of the same sample were highly significant in the case of sample P5L ($p < 0.001$), significant for sample P3L ($p < 0.05$) and insignificant in the case of sample P1L ($p > 0.05$). These results indicate a clear influence of *Cetraria islandica* on the appearance of the samples, especially at concentrations higher than 3%, and also, somewhat limited absorption of the brine solution with lichens to the center of the samples can be observed.

Table 3. Colour parameters of the studied batches (L, a*, b*, h°, C*, ΔE values; mean ± SD; n = 10)

Studied colour parameters		Batches			
		P0L	P1L	P3L	P5L
L*	Subsurface	$64.97 \pm 0.89^{b,A}$	63.69 ± 0.67^{a}	70.73 ± 0.59^{c}	73.73 ± 0.50^{d}
	Interior		67.33 ± 0.46^{B}	68.78 ± 0.53^{C}	71.19 ± 0.46^{D}
a*	Subsurface	$10.56 \pm 0.69^{c,C}$	6.87 ± 2.33^{b}	5.85 ± 0.20^{b}	3.19 ± 0.43^{a}
	Interior		8.38 ± 0.37^{B}	6.80 ± 0.43^{A}	6.40 ± 0.22^{A}
b*	Subsurface	$9.01 \pm 0.37^{a,A}$	10.06 ± 0.65^{b}	11.04 ± 0.25^{c}	11.92 ± 0.42^{d}
	Interior		10.70 ± 0.29^{B}	11.76 ± 0.26^{C}	12.75 ± 0.28^{D}
Hue (h°)	Subsurface	$49.49 \pm 2.37^{c,D}$	33.31 ± 11.15^{b}	27.91 ± 1.16^{b}	15.39 ± 2.18^{a}
	Interior		38.05 ± 1.77^{C}	30.04 ± 2.13^{B}	26.67 ± 0.89^{A}
Chroma (C*)	Subsurface	$13.89 \pm 0.53^{b,AB}$	12.34 ± 1.26^{a}	12.50 ± 0.20^{a}	12.34 ± 0.43^{a}
	Interior		13.60 ± 0.22^{A}	13.59 ± 0.05^{A}	14.27 ± 0.28^{B}
ΔE	Subsurface	-	4.40 ± 2.30^{a}	7.77 ± 0.88^{b}	11.85 ± 0.97^{c}
	Interior		3.72 ± 1.08^{A}	6.10 ± 1.04^{B}	8.41 ± 1.08^{C}
Statistical significance (p-value) between Subsurface*Inner	L*	-	***	***	***
	a*	-	*	ns	***
	b*	-	**	**	***
	Hue (H°)	-	ns	ns	***
	Chroma (C*)	-	***	***	***
	ΔE	-	ns	*	***

P0L - control pork leg (no added lichens); P1L - pork leg with 1% added lichens; P3L - pork leg with 3% added lichens; P5L - pork leg with 5% added lichens.

Values within the same row followed by different lowercase letters (a, b, c, d) differ significantly ($p < 0.05$) for subsurface samples; Values followed by different uppercase letters (A, B, C, D, E) differ significantly ($p < 0.05$) for interior samples.

Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The texture profile analysis (TPA) of the samples, presented in Table 4, highlights their mechanical properties and reveals the influence of *Cetraria islandica* compared to the control sample (without the addition of the lichen).

The textural parameters of pork legs were in most cases significantly affected by the addition of *Cetraria islandica* ($p < 0.05$), according to the results obtained (Table 4).

The Hardness parameter showed significant differences between all the analyzed samples ($p < 0.05$), its values registering more pronounced decreases concomitantly with the increase in the concentration of lichens added to the analyzed samples. The highest value for this parameter was identified in the case of the control sample P0L (21.56 ± 2.20 N), and the lowest in the P5L batch (8.87 ± 0.54 N). These results can be correlated with the higher moisture content of the samples with a higher concentration of lichens which determines a decrease in this parameter. Thus, we can deduce the obvious influence of lichens on the hardness of the samples. The influence of lichens is initially more evident between the P0L-P1L and P1L-P3L samples, showing decreases in the average value of over 5N, and between the P3L-P5L batches the decrease in the hardness of the samples is below 2N. This phenomenon can be attributed to a possible saturation of the meat matrix's capacity to integrate additional available water, suggesting

the existence of an efficiency threshold beyond which the additional addition of lichen no longer produces significant changes in hardness. The statistical differences between each of the samples with *Cetraria islandica* and the control sample are highly significant ($p < 0.001$).

Adhesiveness is the only textural parameter that recorded increases in average values with the increase in the amount of lichen added, which indicates a slight change in the surface interaction of the samples, but without a very perceptible impact from a subjective point of view, as we will see in the sensory analysis, but only in the case of subjective analysis. The control sample P0L and the sample with 1% lichen addition P1L did not identify significant differences ($p > 0.05$), and between P0L and samples P3L and P5L very significant differences ($p < 0.01$) and extremely significant differences ($p < 0.001$) were identified. As for the differences between samples with the addition of *Cetraria islandica*, they are statistically significant only between P1L and P5L ($p < 0.05$).

The elasticity, although it did not register drastic variations, nevertheless showed very significant differences between the control sample and the P3L and P5L samples ($p < 0.001$), showing a decreasing trend with the increase in the addition of lichen, the values decreasing from 0.43 ± 0.03 mJ in the control sample (POL) to 0.32 ± 0.05 mJ in the batch with 5% addition of *Cetraria islandica*. This decrease in elasticity caused by the vegetable addition affecting the protein networks (decrease in protein and collagen content, as can be seen in Table 2) of the samples determines a weaker return of the sample to its initial shape after deformation.

As for cohesiveness, it presents statistical significance only between the control sample and the samples with 3 and 5% addition of *Cetraria islandica*, as in the case of elasticity, the differences being very significant for the P3L sample ($p < 0.01$) and extremely significant for the P5L sample ($p < 0.001$). From these results we can deduce that the structural stability of the product is slightly affected at additions above 3% *Cetraria islandica* which slightly affects the protein content causing slight structural changes.

The gumminess and chewiness of the samples, on the other hand, are extremely significantly affected ($p < 0.001$) by the samples with the addition of *Cetraria islandica* (P1L, P3L and P5L) compared to the control sample (P0L). These, as was also identified for all other textural parameters except adhesiveness, recorded successive reductions in the average values concomitant with the increase in the *Cetraria islandica* content. The lowest values of these two parameters were identified within the batch with 5% addition of *Cetraria islandica*, being 4.72 ± 0.68 N for gumminess and 1.53 ± 0.42 J for chewiness. No significant differences were identified between the P5L and P3L samples in the case of chewiness ($p > 0.05$), but significant differences were identified between these two samples and the P1L sample ($p < 0.05$). In contrast, for the textural parameter gumminess, significant differences were identified between all 3 samples with the addition of *Cetraria islandica* ($p < 0.05$).

Following the analysis of the textural profile of the investigated products (Table 4), it was found that the addition of *Cetraria islandica* causes a significant reduction in most textural parameters, especially hardness and chewiness. These changes suggest an improvement in tenderness, leading to meat products with a softer consistency and easier chewing compared to the control sample (P0L). The effect is observed through reduced values of hardness and chewiness, which decrease with increasing concentration of added lichen. These results correlate positively with consumer research suggesting that tenderness is a very important element of edible quality and that variations in tenderness and chewiness affect the decision to consume and purchase meat (Maltin et al., 2003).

To our knowledge, no other research on the effect of *Cetraria Islandica* on the texture of meat products has been conducted so far.

Table 4. Instrumental texture profile analysis (TPA) of the samples (mean ± SD; n = 10)

Texture profile analysis parameters		Batches			
		P0L	P1L	P3L	P5L
Hardness (N)		21.56 ± 2.20^{d}	16.04 ± 1.02^{c}	10.60 ± 0.88^{b}	8.87 ± 0.54^{a}
Adhesiveness (mJ)		0.02 ± 0.03^{a}	0.03 ± 0.02^{ab}	0.05 ± 0.01^{bc}	0.07 ± 0.02^{c}
Elaticity		0.43 ± 0.03^{c}	0.40 ± 0.03^{bc}	0.35 ± 0.06^{ab}	0.32 ± 0.05^{a}
Cohesiveness		0.65 ± 0.05^{c}	0.62 ± 0.05^{bc}	0.57 ± 0.05^{ab}	0.53 ± 0.07^{a}
Gumminess (N)		14.09 ± 1.54^{d}	10.00 ± 1.10^{c}	6.06 ± 0.93^{b}	4.72 ± 0.68^{a}
Chewiness (J)		6.07 ± 0.87^{c}	3.96 ± 0.55^{b}	2.12 ± 0.58^{a}	1.53 ± 0.42^{a}
Statistical significance (p-value) between the control batch (P0L) and the other batches	Hardness (N)	-	***	***	***
	Adhesiveness (mJ)	-	ns	**	***
	Elaticity	-	ns	***	***
	Cohesiveness	-	ns	**	***
	Gumminess (N)	-	***	***	***
	Chewiness (J)	-	***	***	***

P0L - control pork leg (no added lichens); P1L - pork leg with 1% added lichens; P3L - pork leg with 3% added lichens; P5L - pork leg with 5% added lichens.
Values within the same row followed by different lowercase letters (a, b, c, d) differ significantly ($p < 0.05$) for subsurface samples; Values followed by different uppercase letters (A, B, C, D, E) differ significantly ($p < 0.05$) for interior samples.
Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Existing studies focus mainly on the antioxidant and antimicrobial effects of this plant, omitting its other possible benefits.

The phytochemical composition and antioxidant capacity of pastrami samples with added *Cetraria islandica*, both thermally treated and untreated, are presented in Table 5 for both analyzed regions of the meat pieces (subsurface and center). The phytochemical and antioxidant characterization of *Cetraria islandica* is also presented in order to be able to follow the effect compared to the meat samples. It was found that the addition of lichens does not modify the flavonoid content of the studied samples in any of the analyzed regions, the differences observed being insignificant ($p > 0.05$). The content of phenolic compounds in the interior of the samples presents the same results regarding statistical differences as the content of phenolic compounds. However, in the subsurface region, statistical differences were observed between the control samples and the samples with the addition of lichens, the differences being significant ($p < 0.05$), but between the batches that presented the addition of 1, 3 and 5% *Cetraria islandica*, slight increases in the average values were recorded concomitantly with the increase in the addition of lichens in the samples, especially in the heat-treated samples, but the differences were statistically insignificant ($p > 0.05$). Also, the Tukey test did not register significant differences between the treated and non-heat-treated samples in terms of the content of flavonoids and polyphenols ($p > 0.05$).

In contrast, the antioxidant capacity of the products evaluated by the DPPH and ABTS methods presented very differentiated results between the average results of the samples depending on the concentration of *Cetraria islandica* added and depending on the heat treatment, as can be seen in Table 5. In the case of the DPPH analysis, a successive increase in antioxidant activity was observed concomitant with the increase in the concentration of lichens added both in terms of the amount of µMol Trolox/g DW and, inherently, the inhibition of DPPH oxidation. In the subsurface region, the DPPH value (µMol Trolox/g DW) increased from 0.60 ± 0.43 µMol Trolox/g DW in the non-thermally treated samples to 7.50 ± 0.31 µMol Trolox/g DW in the samples with 5% *Cetraria islandica* addition and in the thermally treated samples from 0.70 ± 0.06 µMol Trolox/g DW to 7.72 ± 0.32 µMol Trolox/g DW. The inhibition of DPPH oxidation (%) showed increases from the P0L control sample value of 1.52 ± 0.70% to 13.65 ± 0.55% in the non-thermally treated samples and from 1.98 ± 0.12% to 16.14 ± 0.65% in the thermally treated samples. In the inner region, the increase in antioxidant activity determined by the DPPH method was observed, but the values were not as high. The values of the control samples in the centeral region were identical to those of the subsurface region, but in this case the average values increased only to

4.73 ± 0.25 µMol Trolox/g DW in the non-thermally treated samples and to 5.82 ± 0.33 µMol Trolox/g DW in the thermally treated samples. Also, the DPPH inhibition was lower in the central region of the samples, with a maximum of 8.52 ± 0.43% in the non-thermally treated samples and 11.94 ± 0.65% in the thermally treated samples.

Table 5. Antioxidant capacity and phytochemical compound content in compact-structured meat products with added *Cetraria islandica* (mean ± SD)

Studied antioxidant characteristics			Batches							
		Cetraria islandica	P0L		P1L		P3L		P5L	
			NT	TT	NT	TT	NT	TT	NT	TT
Total flavonoids (mg CE/g DW)	Subsurface	0.14 ± 0.01^{b}	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}	0.004 ± 0.00^{a}	0.005 ± 0.00^{a}	0.005 ± 0.00^{a}	0.006 ± 0.00^{a}	0.005 ± 0.00^{a}	0.007 ± 0.00^{a}
Total polyphenols (mg AG/g DW)		0.07 ± 0.00^{c}	0.001 ± 0.00^{a}	0.002 ± 0.00^{a}	0.005 ± 0.00^{ab}	0.008 ± 0.00^{ab}	0.006 ± 0.00^{ab}	0.009 ± 0.00^{b}	0.006 ± 0.00^{ab}	0.010 ± 0.00^{b}
DPPH (µMol Trolox/g DW)		4.19 ± 0.01^{c}	0.60 ± 0.43^{a}	0.70 ± 0.06^{a}	1.84 ± 0.07^{b}	4.49 ± 0.71^{c}	4.09 ± 0.59^{c}	5.71 ± 0.15^{d}	7.50 ± 0.31^{e}	7.72 ± 0.32^{e}
DPPH (% Inhibition)		29.89 ± 0.07^{g}	1.52 ± 0.70^{a}	1.98 ± 0.12^{a}	3.62 ± 0.12^{b}	9.76 ± 1.46^{d}	7.58 ± 1.01^{c}	12.21 ± 0.31^{e}	13.65 ± 0.55^{e}	16.14 ± 0.65^{f}
ABTS (mMol Trolox/g DW)		258.56 ± 4.05^{c}	65.09 ± 2.69^{a}	199.08 ± 3.18^{b}	489.22 ± 4.65^{d}	905.20 ± 5.75^{g}	578.36 ± 7.14^{e}	1354.73 ± 6.25^{h}	659.54 ± 3.15^{f}	1605.41 ± 4.39^{i}
ABTS (% Inhibition)		38.41 ± 0.51^{f}	8.38 ± 0.10^{a}	12.94 ± 0.12^{b}	20.40 ± 0.14^{c}	39.25 ± 0.21^{g}	23.64 ± 0.22^{d}	55.79 ± 0.23^{h}	26.46 ± 0.10^{e}	64.38 ± 0.16^{i}
Total flavonoids (mg CE/g DW)	Center	0.14 ± 0.01^{b}	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}	0.004 ± 0.00^{a}	0.004 ± 0.00^{a}	0.005 ± 0.00^{a}	0.004 ± 0.00^{a}	0.005 ± 0.00^{a}
Total polyphenols (mg AG/g DW)		0.07 ± 0.00^{b}	0.001 ± 0.00^{a}	0.002 ± 0.00^{a}	0.003 ± 0.00^{a}	0.003 ± 0.00^{a}	0.004 ± 0.00^{a}	0.005 ± 0.00^{a}	0.004 ± 0.00^{a}	0.006 ± 0.00^{a}
DPPH (µMol Trolox/g DW)		4.19 ± 0.01^{d}	0.60 ± 0.43^{a}	0.70 ± 0.06^{ab}	1.40 ± 0.24^{b}	3.36 ± 0.33^{c}	3.29 ± 0.61^{c}	4.28 ± 0.48^{d}	4.73 ± 0.25^{d}	5.82 ± 0.33^{e}
DPPH (% Inhibition)		29.89 ± 0.07^{g}	1.52 ± 0.70^{a}	1.98 ± 0.12^{ab}	2.87 ± 0.39^{b}	7.27 ± 0.65^{cd}	6.03 ± 1.01^{c}	8.98 ± 0.95^{e}	8.52 ± 0.43^{de}	11.94 ± 0.65^{f}
ABTS (mMol Trolox/g DW)		258.56 ± 4.05^{d}	65.09 ± 2.69^{a}	199.08 ± 3.18^{c}	167.21 ± 8.56^{b}	715.20 ± 5.77^{g}	309.58 ± 2.02^{e}	885.65 ± 5.15^{h}	381.44 ± 4.13^{f}	956.29 ± 3.32^{i}
ABTS (% Inhibition)		38.41 ± 0.51^{h}	8.38 ± 0.10^{a}	12.94 ± 0.12^{c}	10.57 ± 0.26^{b}	31.52 ± 0.21^{f}	14.92 ± 0.06^{d}	37.22 ± 0.18^{g}	17.23 ± 0.13^{e}	39.56 ± 0.12^{i}
Statistical significance (p-value) between Thermal treatment within the same region (subsurfaceor interior)	Total flavonoids	Subsurface	-	-	ns		ns		ns	
		Center			ns		ns		ns	
	Total polyphenols	Subsurface			ns		ns		ns	
		Center			ns		ns		ns	
	DPPH	Subsurface			***		***		ns	
		Center			***		**		**	
	DPPH (%Inhibition)	Subsurface			***		***		***	
		Center			***		***		***	
	ABTS	Subsurface			***		***		***	
		Center			***		***		***	
	ABTS (%Inhibition)	Subsurface			***		***		***	
		Center			***		***		***	

Nt – not-thermally treated; TT – thermally treated

P0L - control pork leg (no added lichens); P1L - pork leg with 1% added lichens; P3L - pork leg with 3% added lichens; P5L - pork leg with 5% added lichens.

Values within the same row followed by different lowercase letters (a, b, c, d) differ significantly ($p < 0.05$) for subsurface samples; Values followed by different uppercase letters (A, B, C, D, E) differ significantly ($p < 0.05$) for interior samples.

Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Statistically, the differences in DPPH antioxidant capacity in terms of the amount of µMol Trolox/g DW depending on the presence or absence of heat treatment were highly significant ($p < 0.001$) for samples with 1% addition of *Cetraria islandica* in both regions and in the region with 3% addition in the subsurface region, and in the interior region for the same concentration very significant differences were observed ($p < 0.01$), as in the case of the same region at the concentration of 5% addition of lichens. In contrast, at this concentration, but in the subsurface region, insignificant differences were observed between the treated and untreated samples ($p > 0.05$).

Results with the same trends as DPPH were also observed in the case of ABTS analysis, except that the latter recorded higher average values of antioxidant capacity, demonstrating its greater compatibility with the food matrix used (meat), also having a wider applicability than the DPPH method which allows the identification of a more developed spectrum of

compounds with antioxidant action. The antioxidant activity values evaluated by the ABTS method in the subsurface region, recorded within the experimental batches maximum values in the batches with 5% addition of lichens of 1605.41 ± 4.39 mMol Trolox/g DW and inhibition of 64.38 ± 0.16% in the heat-treated batch, which even exceeds the antioxidant capacity of *Cetraria islandica* which shows an inhibition of only 38.41 ± 0.51%. Also, the batches with 1 and 3% addition of heat-treated *Cetraria islandica* show a higher oxidation inhibition capacity than *Cetraria islandica* of 39.25 ± 0.21% and, respectively, 55.79 ± 0.23% and an activity of 905.20 ± 5.75 mMol Trolox/g DW and, respectively, 1354.73 ± 6.25 mMol Trolox/ g DW.

The non-thermally treated samples, on the other hand, present an ABTS antioxidant activity lower on average by approximately 520 mMol Trolox/g DW and an inhibition lower by approximately 28% compared to the thermally treated samples, being obtained for the samples with 1, 3 and 5% addition of *Cetraria islandica* the average antioxidant activity value of 489.22 ± 4.65 mMol Trolox/g DW, 578.36 ± 7.14 mMol Trolox/g DW and, respectively, 659.54 ± 3.15 mMol Trolox/g DW, with an inhibitory capacity of 20.40 ± 0.14%, 23.64 ± 0.22% and, respectively, 26.46 ± 0.10%, values which, in contrast to the thermally treated batches, do not exceed the inhibitory capacity of *Cetraria islandica* but only that of the control batches. (untreated and heat-treated), which obtained antioxidant activity values of 65.09 ± 2.69 mMol Trolox/g DW in the non-heat-treated batch and 199.08 ± 3.18 mMol Trolox/g DW in the heat-treated batch, corresponding to inhibitions of 8.38 ± 0.10% and, respectively, 12.94 ± 0.12%.

All batches analyzed in the case of ABTS analysis (antioxidant activity and oxidation inhibition) showed significant differences between them ($p < 0.05$) both in the subsurface region and in the central region. In the center of the meat pieces, the ABTS analysis values, as also happened in the case of DPPH analysis, were much lower than those in the subsurface region, also demonstrating the limited absorption of the *Cetraria islandica* brine solution due to its viscosity which is higher than a regular brine. The maximum values in this case were also obtained in the batch with 5% addition of *Cetraria islandica*, but which, in this case, were much lower, registering an antioxidant activity of 956.29 ± 3.32 mMol Trolox/g DW in the heat-treated batch and 381.44 ± 4.13 mMol Trolox/g DW in the non-heat-treated batch and an oxidation inhibition capacity of 39.56 ± 0.12% and 17.23 ± 0.13%, respectively.

The batches with 1 and 3% addition of *Cetraria islandica* recorded slightly lower values compared to the batch containing this addition in a percentage of 5%, the minimum (in a batch with *Cetraria islandica*) was observed in batch P1L, which presented an antioxidant activity of 715.20 ± 5.77 mMol Trolox/g DW in the heat-treated batch and of 167.21 ± 8.56 mMol Trolox/g DW in the non-heat-treated batch and an oxidation inhibition capacity of 31.52 ± 0.21% and, respectively, 10.57 ± 0.26%. The results obtained show that the addition of *Cetraria Islandica* also presents an antioxidant effect inside the meat pieces, but not as strong as in the subsurface region.

However, the results of the P5L batch (central region) show an oxidation inhibition capacity that is superior to *Cetraria islandica* and the P3L batch (interior) has a very similar inhibition value. Regarding the statistical differences between the heat-treated and untreated samples within the same region, highly significant statistical differences ($p < 0.001$) are observed between all the batches studied.

The results obtained show that the bioactive compounds in lichens are thermally stable, not being susceptible to thermal degradation under the specific treatments for the production of meat products with a compact structure (which do not fall into the category of raw-dried products). Thermal treatment even helps to release and activate compounds with antioxidant functions from the vegetable addition, probably by breaking the bonds between them and the structural components. Our results are consistent with those reported by Gülçin et al. (2002), highlighting that *Cetraria islandica* has antioxidant activity and potential for use in the food industry. In the present study, it was demonstrated that this lichen can be successfully applied in the meat

industry, due to its resistance to the temperatures specific to the thermal treatments used in meat processing.

Similar results were reported by Krotova et al. (2023), who added a 1% vegetable extract of milk thistle and Icelandic moss to the composition of minced meat semi-finished products, referred to as "Special cutlets". As in our study, they observed an improvement in the antioxidant value of the products without negatively affecting their sensory properties. For batch P1L, with the addition of 1% *Cetraria islandica*, we also obtained similar results in the sensory analysis, the studied characteristics not being negatively affected by the vegetable addition, as we can see in Table 6 and Figure 1. However, the batches with the addition of 3 and 5% lichen suffered sensory changes that led to their low acceptability among consumers.

Table 6. Sensory characteristics of the experimental pastrami batches with *Cetraria islandica* addition (Mean ± SD; n = 12)

Sensory attributes	Samples				*p*-value
	P0L	P1L	P3L	P5L	
Colour uniformity	8.08 ± 0.90	7.83 ± 0.84	7.33 ± 0.99	6.75 ± 1.29	0.013 (*)
General aspect	8.58 ± 0.52	7.83 ± 0.94	6.92 ± 0.67	6.83 ± 0.94	0.000 (***)
Specific smell of meat	8.67 ± 0.65	8.42 ± 0.79	8.17 ± 0.84	7.67 ± 0.89	0.025 (*)
Smoky odor	7.50 ± 0.80	7.42 ± 1.17	7.25 ± 1.14	7.33 ± 1.07	0.945 (ns)
Plant odors	1.08 ± 0.29	1.33 ± 0.65	1.67 ± 0.65	2.33 ± 0.78	0.000 (***)
Foreign odors for meat products	1.17 ± 0.39	1.42 ± 0.67	1.67 ± 0.78	2.00 ± 1.04	0.060 (ns)
Meat odour intensity	5.75 ± 0.75	5.33 ± 0.89	4.75 ± 0.75	4.25 ± 1.06	0.001 (***)
Unpleasant odor	1.00 ± 0.00	1.00 ± 0.00	1.17 ± 0.39	1.25 ± 0.45	0.113 (ns)
Global odour perception	8.25 ± 0.62	8.00 ± 0.95	7.75 ± 0.97	7.17 ± 1.03	0.035 (*)
Specific meat product taste	8.92 ± 0.29	8.50 ± 0.80	7.92 ± 0.80	6.67 ± 0.49	0.000 (***)
Salty taste	5.50 ± 0.67	5.08 ± 0.67	4.75 ± 0.62	4.17 ± 0.72	0.000 (***)
Smoky taste	5.00 ± 0.74	4.58 ± 0.52	4.25 ± 0.62	3.67 ± 0.78	0.000 (***)
Bitter taste	1.08 ± 0.29	1.17 ± 0.39	1.58 ± 0.52	2.08 ± 0.52	0.000 (***)
Taste persistence	6.58 ± 0.79	6.75 ± 0.75	6.83 ± 0.72	6.50 ± 0.80	0.703 (ns)
Taste balance	7.75 ± 0.87	6.92 ± 0.79	5.75 ± 0.87	5.00 ± 0.74	0.000 (***)
Tender	5.75 ± 0.62	5.83 ± 0.72	6.08 ± 0.67	6.25 ± 0.62	0.239 (ns)
Juiciness	5.67 ± 0.78	5.75 ± 0.62	6.00 ± 0.60	6.08 ± 0.67	0.381 (ns)
Grany texture	1.42 ± 0.67	1.75 ± 1.06	2.25 ± 1.14	2.42 ± 1.00	0.062 (ns)
Firmness	5.25 ± 0.87	4.92 ± 1.00	4.58 ± 0.79	4.50 ± 0.80	0.148 (ns)
Overall product acceptability	8.33 ± 0.65	7.75 ± 0.62	7.33 ± 0.99	6.50 ± 0.80	0.000 (***)

P0L - control pork leg (no added lichens); P1L - pork leg with 1% added lichens; P3L - pork leg with 3% added lichens; P5L - pork leg with 5% added lichens.
Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 6 presents the sensory characteristics evaluated by tasters during the sensory analysis of pastrami batches with the addition of *Cetraria islandica*. The products were affected by this addition especially in terms of sensory characteristics related to the appearance, smell and taste of the samples.

The appearance of the samples, characterized by the characteristics of colour uniformity and general aspect, showed significant differences ($p < 0.05$) and, respectively, highly significant ($p < 0.001$) between the analyzed samples, registering lower average scores in batches P3L and P5L compared to batches P0L (without addition of lichens) and P1L (the batch with the lowest concentration of lichens). These results are most likely caused by the color changes that are quite evident in the batches with 3 and 5% addition of lichens, as highlighted by the instrumental color analyses in Table 3 and the non-uniform dispersion of lichen in the product mass.

From the olfactory side of the sensory analysis, the appearance of plant odour was observed, which caused very significant differences between the samples ($p < 0.001$), which also caused a decrease in meat odour intensity and specific meat product taste, with extremely significant differences between the samples analyzed ($p < 0.001$). However, these changes in odour were not considered unpleasant, since the Unpleasant odour attribute did not present statistical differences between the samples studied ($p > 0.05$).

The taste was most strongly affected by the addition of lichens, leading to a decrease in the specific taste of meat, salt and smoke and to an increase in bitter taste, with extremely significant differences between samples ($p < 0.001$). The taste balance of the products was also greatly affected by the vegetable addition, higher concentrations of the addition leading to a significant decrease in it. Most likely, the bitter taste induced by the addition of lichens contributed to a decrease in perceived balance and overall hedonic response, reflected in our study by the parameter "Overall product acceptability". This attribute showed an extremely significant reduction in scores ($p < 0.001$), particularly in the samples with higher lichen concentrations (P3L and P5L, 3% and 5%, respectively).

The parameters referring to the texture of the samples were not significantly affected by the addition of lichens, presenting insignificant differences between batches ($p > 0.05$). However, a positive trend of improving the tenderness and juiciness of the products by increasing the addition of *Cetraria islandica* is observed, which confirms the results obtained by us in the objective analysis of the texture (Table 4).

Figure 1 shows the PrefMap (preference map) that graphically shows where the four pastrami samples analyzed in this study (P0L, P1L, P3L and P5L) are located. As we can see, the samples P0L and P1L are located approximately in the same color area of the graph, which denotes a similar preference for them.

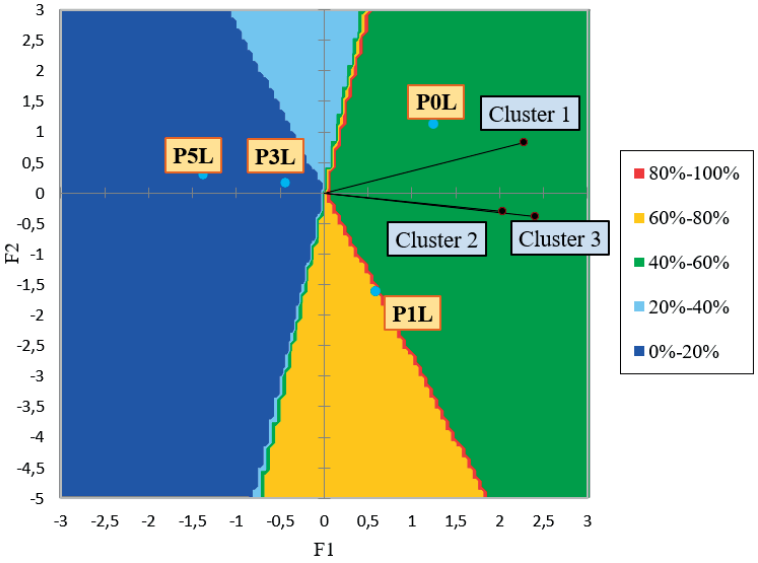


Figure 1. PrefMap for sensory profile and assessor preferances for the experimental batches
P0L - control pork leg (no added lichens); P1L - pork leg with 1% added lichens; P3L - pork leg with 3% added lichens; P5L - pork leg with 5% added lichens; Clusters indicate groups of consumers with similar preferences towards the analyzed samples

Also, the three clusters of tasters are located only in this part of the graph, being close to each other, which shows that the preferences of all participants in the sensory analysis were aligned with each other, which indicates a common preference. As we can observe in Figure 1, the samples with the highest addition of *Cetraria islandica*, P3L and P5L are located on the left of the graph, in the dark blue area, which denotes a low preference for these products, below 20%, which correlates with the hedonic score. Overall product acceptability present in table 6 and shows that, among tasters, products with 3 and 5% addition of lichens have a low acceptability. The sample with 1% addition of lichens, on the other hand, enjoys good acceptability among consumers, being located between the red and yellow areas, which indicates an acceptability of approximately 80% among tasters.

The green area of the graph, as we can see, is not identical to the one in the legend, which indicates that it is an overlap of two colors in the graph (most likely red and green), meaning that it belongs to an intermediate preference, but not in a certain score of a color in the legend. This shows that the tasters' preferences for the samples located in this area were not identical, but not in contradiction either, there being no polarization of the product scores, since they were, however, coherent.

CONCLUSIONS

The physicochemical results show that the addition of lichens leads to a better water retention capacity in the samples and an increase in their tenderness. A reduction in fat and salt content was also observed, which corresponds to current consumer requirements.

The evaluation of the antioxidant capacity of experimental meat samples enriched with *Cetraria islandica* suggests that this lichen can be successfully used in compact meat products to enhance their phytochemical profile. Notably, the antioxidant activity of these products is further increased by the application of heat treatment, which is essential for producing market-ready formulations. Our results use this lichen as a functional ingredient in innovative meat products, because it possesses a nutritional value created compared to a conventional one, high antioxidant properties and good potential for prolonging the oxidative stability of meat products such as pastrami.

Unfortunately, homogeneous penetration to the center of the pieces is not possible due to the high viscosity of the solution containing *Cetraria islandica*, which limits diffusion into the meat mass. This unequal distribution affects the product's functional and qualitative homogeneity by determining notable differences in the physicochemical and phytochemical parameters between the core and peripheral regions. The uniform penetration of the lichen solution into the meat mass may be enhanced by the use of micronized powders or sophisticated injection technologies, which would help produce products with consistent functional qualities.

In contrast, consumers do not particularly prefer products with higher additions of lichens, 3 and 5%, due to the modified characteristics that distance them from the control batch and, inherently, from the classic meat products found on the market, this fact slightly affecting the sensory acceptability of the products.

Our results highlight the need to identify an optimal balance between the functionality and acceptability of new products, in order to develop products that meet both the nutritional and sensory requirements of consumers.

All the results obtained in our study show that *Cetraria islandica* could be successfully utilized in the food industry in terms of improving the physical and biochemical quality of meat products with compact structure. Higher inclusion levels, however, have a detrimental effect on the products sensory quality, as demonstrated by our research. Nonetheless, moderate levels are well accepted by consumers and contribute to an overall improvement in both the sensory quality and antioxidant potential of the final products.

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QUALITY CHARACTERISATION AND CONSUMER PERCEPTION OF A NOVEL FUNCTIONAL BEEF SNACK ENRICHED WITH MACA (*LEPIDIUM MEYENII*)

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Abstract

Maca is a plant-based superfood that enjoys great popularity for its bioactive effects. Although the benefits of its use are well documented in peer-reviewed articles, studies on its addition to meat are extremely limited, with almost no research on adding the superfood maca to meat products. Considering the popularity of snacks among the younger generation, we thought to create such a food that represents a quick and healthy alternative to classic snacks by enriching beef with maca. This innovative alternative increases the antioxidant capacity of beef, providing a nutritious and attractive food for today's consumers. In order to evaluate the impact of maca in beef snacks, we made 3 batches, one without maca addition and 2 batches with 1 and 3% maca addition. These were subjected to detailed physico-chemical and sensory analyses to characterize the product in terms of quality and consumer acceptability. The addition of maca (Lepidium meyenii) to beef snacks resulted in a clear improvement in antioxidant capacity, with DPPH and ABTS inhibition values increasing progressively with the level of plant addition concentration. The maca samples exhibited a higher antioxidant capacity than the control (BSM0), with DPPH inhibition reaching up to 37.07 ± 0.13% (vs. 17.75 ± 0.23%) and ABTS up to 39.13 ± 0.15% (vs. 19.41 ± 0.11%) in the 3% maca batch, together with a significantly higher content of polyphenols and flavonoids. From a sensory point of view, the 1% maca batch (BSM1) maintained a very good acceptability, comparable to the control, with only minor changes in the sensory profile. The data obtained were statistically analyzed in order to identify significant differences and to determine the influence of maca concentration on the finished products.

Key words*: antioxidants, food innovation, functional meat products, sensory analysis, superfood.*

INTRODUCTION

Over the centuries, we have evolved in the way we look at food. In the beginning, people saw food as a way to get the energy and nutrients the body needed. As the science of nutrition progressed, it was later recognized as a way to promote healthy growth and development. Nowadays, food is also considered to play a significant role in preventing certain diet-related diseases (Pogorzelska-Nowicka et al., 2018).

The development of functional meat products that exhibit health benefits and possess improved overall quality have received much attention in recent years (Zhang et al., 2010). By reformulating food preparations with bioactive components, the aim is to improve the physiological activity of natural nutrients and meet consumer demand for the development of value-added products (Ciobotaru et al., 2024a).

First used in Japan in the mid-1980s, the phrase "functional foods" describes processed foods that include nutrients together with additives (natural or synthetic) that support certain physiological processes (Anchidin et al., 2024b).

A common and straightforward definition of "functional foods", despite the lack of a universally accepted definition, is "processed foods that have disease prevention and/or health-promoting benefits in addition to their nutritional value" (Arihara & Ohata, 2011).

Functional compounds are added to foods to enhance their nutritional value and possible health benefits. Enriching the original products with health-promoting elements is the foundation of the concept of producing foods with a greater beneficial influence on health.

It appears that animal products and meat are an excellent matrix for creating new products with improved value (Pogorzelska-Nowicka et al., 2018). Also, modern food processing involves the use of various food improvement agents (Zugravu et al., 2017).

In the most recent Statistical Yearbook on Food and Agriculture, the FAO reported that 360.618 million tons of meat were produced in 2022 compared to 231.912 million tons in 2000, this highlights a 55.5% increase in meat production over the last two decades (FAO, 2024). This is largely due to population and income growth (Gucianu et al., 2024), but also to the fact that in contemporary society, meat and animal products provide a certain type of culinary satisfaction (Ciobanu et al., 2024). The meat industry is one of the most important in the world and, due to strong consumer demand and intense rivalry, there is a continuous demand for new product development (Boișteanu et al., 2025). In addition to this aspect, we can also add the consumers' increased demand on the food they buy and consume (Pogurschi et al., 2018) which leads to the need to respond to their demands through innovation.

Because they include proteins with high biological value, fat-soluble vitamins, minerals, trace elements and bioactive substances, meat and meat products are both nutrient-rich and diverse (Manoliu et al., 2024) and are recognized as essential foods for human consumption (Boișteanu et al., 2023; Ciobanu et al., 2023a; Anchidin et al., 2024a; Flocea et al., 2024). They perform various biological functions in the human body (Manoliu et al., 2023) that are of great interest nowadays due to the increasing concern of people about the health benefits they possess (Anchidin et al., 2023). The increase in meat production and consumption is due, in addition to the need to provide food for a growing population, to its high nutritional and biological value, especially as a good source of complete protein, with meat traditionally associated with health and prosperity (Ciobanu et al., 2025; Ciobotaru et al., 2024b).

Among other bioactive substances, meat products are excellent suppliers of iron, zinc, conjugated linoleic acid (found mainly in ruminants) and B vitamins. One of the most nutrient-rich and prized foods is beef. The importance of beef as a source of micronutrients (such as vitamins A, B6, B12, D, E, iron, zinc and selenium) and high biological value protein is widely recognized (Scollan et al., 2006). Despite all these beneficial characteristics, meat and meat products are perishable products that need additives to prevent them from spoiling quickly. One of the main reasons why meat and meat products lose their quality is lipid oxidation (Gucianu et al., 2023). As a result of these processes, meat products lose some of their nutritional content and sensory appeal. The changes caused by oxidation can affect product safety (hazardous chemicals) or even consumer acceptance (changes in color and texture, as well as rancid taste and smell). Antioxidants are added in this context to delay or prevent the occurrence of these negative effects (Pateiro et al., 2021).

The addition of plant-based, antioxidant ingredients that may be beneficial to health could further improve the nutritional profile of meat products and reduce the risk of quality impairment. These products fall into the category of functional foods, which are foods with higher nutritional profiles than conventional products (Decker & Park, 2010). And the addition of natural and organic additives to enhance their quality would align with new market trends (Grigore et al., 2023). The use of natural ingredients for meat preservation and enhancement, such as antioxidants, vitamins, minerals or fibers derived from plants and plant materials with a high content of bioactive components (such as phenolic compounds), has gained in popularity among consumers (Karakaya et al., 2011). One such type of ingredient with demonstrated antioxidant activity is Maca (*Lepidium meyenii*) (Merzah, 2022).

Maca (*Lepidium meyeni; Lepidium peruvianum* is a synonym), probably cultivated on the high plateaus of the central Andes of Peru between 4000 and 1200 BC (Wang & Zhu, 2019) is used as a dietary supplement and for its health benefits. Different varieties of maca are available in a range of 13 colors, from black to yellow. Experimental research shows that maca has a positive impact on mood, memory, fertility and nutrition (Gonzales et al., 2009).

The edible part of the plant, the tuber, is highly nutritionally interesting compared to other root crops such as turnips, potatoes and carrots. The additional nutritional components of dried and preserved maca tubers are similar to those of the seeds and legumes (rice, maize and corn) of other plants. Maca is composed of 59% carbohydrates, 2% lipids and 10% protein (Dini

et al., 1994). Maca is rich in fiber and other minerals such as iron, copper, and vitamin C. In addition to these vital nutrients, this root has bioactive components that help those trying to find a balanced diet (Peres et al., 2020). The presence of bioactive substances including glucosinolates and flavonoids in maca (*Lepidium meyenni*) flour has positive health effects. Antioxidants derived from maca can also be used to regulate enzymatic browning of fresh produce and prevent rancidity in high-fat diets (Cerrón-Mercado et al., 2022). It is one of the most therapeutic plants, rich in antioxidants that protect cells from mutations and damage caused by free radicals. In addition, it contains large amounts of vitamins and is very rich in flavonoids that protect against many diseases (Merzah, 2022). Research by Sandoval et al. (2002) showed that maca has the ability to scavenge free radicals, reduce peroxynitrite-induced cell death, and protect cells from hydrogen peroxide by maintaining intracellular ATP production. All these results suggest that maca may scavenge free radicals and provide cytoprotection against oxidative stress.

Due to its numerous purported health benefits and its prominent and ubiquitous biological functions, maca continues to be of significant practical and scientific importance (*Lepidium meyenii*), becoming a popular functional plant food (Wang & Zhu, 2019; Valentová & Ulrichová, 2003). On top of that, it has been used for centuries in the Central Andes of Peru and no adverse effects have been documented if ingested after boiling. Data from previous reviews of in vivo and in vitro experiments with maca indicate that its use is safe (Valerio & Gonzales, 2005). In the last 5 years, various developed countries in North America, Europe and Asia and beyond have shown interest through an increasing demand for maca (Sandoval et al., 2002) due to its chemical composition, pharmacological effects and positive effects on different metabolisms, with a high demand worldwide in recent times (Korkmaz et al., 2018).

The aim of this research was to develop and characterize a functional beef snack fortified with maca (*Lepidium meyenii*). The study evaluated the influence of the addition of maca at concentrations of 1 and 3% in meat products on the physico-chemical parameters, antioxidant capacity and sensory acceptability of the product, in comparison with a control product (without maca addition).

MATERIALS AND METHODS

For this study, three batches of beef products were manufactured: a control batch, without the addition of maca, and two batches with the addition of 1 and, respectively, 3% yellow maca root powder (*Lepidium meyenii*). These were made in the microproduction workshop for meat and meat products processing of IULS. The raw material was represented by beef leg and beef shoulder, which were purchased from Selgros Cash & Carry SRL in Iași, as well as by semolina flour from Molisana, which was purchased from a local store in Iași. The bioactive yellow maca root powder was purchased from the company Niavis (SC Bio Niavis Trade SRL, Brașov, Romania). The seasoning ingredients were purchased from a local company in Iași county (SC Rocas FDS SRL, Iași, Romania).

The experimental batches and the ingredients used in them are presented in Table 1, in percentage form.

Table 1. Formulation of the experimental batches

Batch code	Raw materials and ingredients (%)											
	Beef round	Beef chuck	Semola flour	Maca root powder	Salt	Sweet paprika	Smooked paprika	Garlic powder	Ground pepper	Onion powder	Ground cumin	Chili flakes
B1M0	53.52	34.05	9.73	-	1.40	0.30	0.20	0.30	0.20	0.20	0.07	0.03
B2M1	52.97	33.70	9.63	1.00								
B3M3	51.87	33.00	9.43	3.00								

B1M0 – control beef snack batch (without maca); B2M1 – beef snack batch with 1% maca; B3M3 – beef snack batch with 3% maca.

The technological process consisted of blending the raw materials and ingredients, coarse grinding, through the intermeid of the meat, followed by its fine grinding in the cutter. During this stage, the spice mixture specific to each batch and the semolina flour were added,

according to Table 1. The resulting composition was introduced into the dosing machine, and by means of a syringe with a diameter of 18mm and a length of ~20 cm, the meat sticks/snacks with/or without the addition of maca were formed, which were placed on the stainless steel grill of the trolleys with racks, in order to perform the heat treatment.

The heat treatment was identical for all three batches and consisted of four stages, as follows: I. drying I of the samples for 30 minutes at 60°C; II. smoking at 70°C for 35 minutes; III. boiling the samples until reaching a temperature of 72°C in the thermal center; IV. second drying for 90 minutes at 75°C. After completing all stages of thermal treatment, the samples were placed in cold storage until cooled, after which they were vacuum packed and stored at 2-4°C for laboratory analysis.

The laboratory analyses carried out in this study consisted of physicochemical evaluations (pH, color parameters, textural parameters, protein content, fat, moisture, dry matter, ash, collagen), analyses of the content of bioactive compounds (antioxidant capacity), as well as sensory analysis. The pH measurements of the experimental batches were performed with the HI98163 pH meter with FC2323 electrode (SC Hanna Instruments SRL). The colorimetric analyses were performed using the Konica Minolta CR-410 colorimeter (Konica Minolta, Inc.) and consisted of the analysis of color parameters in the three-dimensional CIELAB space. Based on the data obtained from these parameters, the colorimetric parameters Hue (H°) and Croma (C*) were also calculated, using formulas (1), (2) and (3), according to the model of Salueña et al. (2019). The textural analysis of the samples was performed using the TA1+1K Plus texturometer (Ametek, Inc., USA), using a cylindrical probe specific to the TPA (Texture Profile Analysis) test, to evaluate the mechanical properties of the double compression type.

$$H° = \arctan \frac{b^*}{a^*} \quad (1)$$

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (2)$$

$$E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (3)$$

Using the Food Check meat analyzer (Bruins Instruments GmbH, KPM Analytics), a flexible near-infrared (NIR) spectroscopic determination method, crude chemical determinations were used to investigate the quantitative analysis of moisture content, protein content, collagen, fat content and salt content.

To perform the antioxidant capacity of the targeted samples, the first stage consisted of the extraction of bioactive compounds. In order to perform this extraction of bioactive compounds from maca root powder and meat samples, one gram of sample was weighed and combined with 10 mL of solvent (70% ethanol). The mixture thus obtained was subjected to ultrasound treatment for 40 minutes, at a controlled temperature of 36 ± 2°C, with a frequency of 40 kHz. The resulting extract was subjected to centrifugation for 10 minutes at 6000 rpm and 10°C. The supernatant obtained after this stage was collected and used in the analyses for phytochemicals (carotenoids, flavonoids and polyphenols) and for the analyses related to antioxidant activity (DPPH and ABTS).

The Folin-Ciocalteu method was used to determine the total polyphenol content, as explained by Trifunschi et al. (2017).

The free radical scavenging activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay in accordance to Trifunschi et al. (2022).

The ABTS assay was used concurrently according to the protocol described by Dumitrașcu et al. (2022).

The absorbance of the meat samples was measured using a UV-Vis spectrophotometer (Specord Plus 210, Analytik Jena, Germany). After vortex mixing, sampling was performed to ensure reproducibility. Flavonoid content was expressed as mg/g DW, polyphenol content as mg GAE/g DW, DPPH and ABTS radical scavenging activity as % inhibition, total carotenoids as mg CT/g DW, and lycopene and β-carotene as mg/g DW. All reagents and standards used for the antioxidant assays were

purchased from Sigma-Aldrich (Steinheim, Darmstadt, Germany).

In addition to all the objective analyses described above, sensory analysis of the samples was also performed (this represents a subjective analysis). This was performed on a panel of 20 semi-trained tasters (13 women and 7 men). We decided to perform this analysis because in the late 1990s, consumer acceptance was cited as the main success factor for functional foods and as the main area of interest for further studies. Numerous studies have demonstrated that consumer acceptance of functional foods is far from unconditional, taste being one of the main requirements for acceptability, in addition to the veracity of health claims (Verbeke et al., 2006). Sensory evaluation of foods involves analyzing consumer reactions based on the senses (smell, appearance, taste, touch and hearing) and has a great influence on the overall quality of the product (Ciobanu et al., 2023b). The analysis was performed 48 hours after the experimental batches were made. The analysis was performed blindly, the samples being coded with three random digits. In this case, the external PrefMap analysis was used, which is an advanced sensory analysis that works with multivariate data, establishing correlations between quantitative data of sensory attributes and consumer preferences (Boișteanu et al., 2025).

Each physico-chemical and biochemical determination was carried out in five independent replicates and the sensory analysis was conducted in a single tasting session.

The following software packages were used for statistical analysis: SPSS (v. 26, IBM Corp., USA) was employed to evaluate the significance of differences between the studied samples based on physicochemical parameters, while XLSTAT (v. 2024.3, Addinsoft, France), used during the free trial period, was applied for sensory analysis using the External Preference Mapping (PrefMap) method.

RESULTS AND DISCUSSIONS

The chemical parameters studied and the pH of the maca-added meat snack samples are presented in Table 2 (mean ± SD) compared to the control lot, which does not have maca (*Lepidium meyenii*) added. The statistical differences between the 3 groups were analyzed by their total (The global p-value considering all samples), as well as by their pairwise comparison (BSM0 vs. BSM1, BSM0 vs. BSM3, BSM1 vs. BSM3).

The average results of the physico-chemical parameters studied showed highly significant differences ($p < 0.01$) for fat, moisture and dry matter content and very highly significant ($p < 0.001$) for the other parameters analyzed (protein, collagen, salt, pH) at their total (global) level (among all the batches evaluated).

The fat content of the samples decreased progressively as the percentage of *Lepidium meyenii* increased, from a maximum of 22.7 ± 0.46% in BSM0 to 21.7 ± 0.22% in BSM3, the lot with 3% added maca (the highest concentration in our study). Although the differences between the BSM0 (control) and BSM1 (1% maca-added) groups were not statistically significant, the BSM3 (3% maca-added) group showed highly significant differences ($p = 0.003$) from the BSM0 group and significant differences (0.015) from the BSM1 group. This reduction in lipid content can be considered as positive, as they are in line with current consumer demands for reduced fat content in meat products, as the growing awareness of the link between diet and health has changed consumer preferences, so that there is an increasing demand for low-fat meat products (Colmenero, 2000).

The moisture content of the samples also showed highly significant decreases ($p = 0.006$) among all samples analyzed, which correlates with the increase in mean dry matter values in the maca fortified batches compared to the control batch, the differences between all of them highly significant ($p < 0.001$). As in the case of fat content, no significant differences ($p > 0.05$) between BSM0 vs BSM1, very highly significant ($p < 0.001$) between BSM0 and BSM3 and only significant ($p < 0.01$) between BSM1 and BSM3 were observed for moisture and dry matter content.

The results obtained for the moisture parameter are at odds with those observed by Cerrón-Mercado et al. (2022) who introduced maca into some burger samples via a gelled emulsion. The addition of maca to these led to an increase in moisture in both raw and cooked samples. The authors do not mention maca as the main factor that led to this increase in moisture, but the water

intake used to create the gel emulsions. However, Alarcón-García et al. (2020) state that maca has a high capacity to retain water in the system in which it is added, being able to reduce moisture losses. However, this effect was not observed in our study, probably due to the fact that maca was incorporated as a dry powder without being dispersed in an aqueous medium.

Protein and collagen contents were decreased by maca additions, with the highest levels of protein and collagen being identified in the control group at 17.26 ± 0.11% and 15.30 ± 0.10%, respectively. The lowest values were observed in the BSM3 group, being 16.26 ± 0.05% and 14.28 ± 0.15%, respectively, which differed very highly significantly ($p < 0.001$) from those of the BSM0 group. This reduction in the protein and collagen content of the samples can be attributed to the addition of maca, which is a plant food, with a low protein content compared to meat, which led to a reduction in the overall value of these two parameters. Between the 1% maca-added group (BSM1) and the control group (BSM0) the differences were significant ($p < 0.05$) for protein content and insignificant ($p = 0.169$) for collagen content. Differences overall (among all 3 samples studied) were very highly significant ($p < 0.001$) for these two parameters (protein and collagen).

Very highly significant differences ($p < 0.001$) in the mean values of the salt parameter were observed between the control sample (BSM0) and the maca samples (BSM1 and BSM3), which showed significant decreases with the addition of maca. However, the interaction between the two maca-added groups (BSM1 and BSM3) was non-significant ($p = 0.131$), indicating that the amount of maca added does not necessarily influence maca content and its presence in a food system.

Maca had a rather significant influence on the acidity of the samples, with very highly significant differences ($p < 0.001$) between all analyzed samples. The decrease in pH (increase in acidity of the samples with maca may be motivated by the acidic character of this plant (its aqueous extracts are acidic), since the soil for its growth must be rich in organic matter and acidic (Gonzales et al., 2009). Also, the acidity of the samples increases significantly ($p < 0.05$) with increasing maca addition, as observed by analyzing the differences between BSM1 and BSM3 plots (Table 2).

Table 2. Physicochemical characteristics of maca-enriched beef snacks

Maca-enriched beef snacks	Studied parameters						
	Fat (%)	Moisture (%)	Dry Matter (%)	Protein (%)	Collagen (%)	Salt (%)	pH
BSM0	22.7 ± 0.46	58.38 ± 0.20	41.62 ± 0.20	17.26 ± 0.11	15.30 ± 0.10	1.54 ± 0.05	6.45 ± 0.10
BSM1	22.48 ± 0.38	58.2 ± 0.43	41.8 ± 0.43	17.02 ± 0.16	14.94 ± 0.48	1.28 ± 0.08	6.23 ± 0.41
BSM3	21.7 ± 0.22	57.64 ± 0.21	42.36 ± 0.21	16.26 ± 0.05	14.28 ± 0.15	1.18 ± 0.08	6.09 ± 0.03
				p-value			
BSM0 vs. BSM1	0.627 ^{ns}	0.621 ^{ns}	0.621 ^{ns}	0.020 *	0.169 ^{ns}	0.000 ***	0.001 ***
BSM0 vs. BSM3	0.003 **	0.006 **	0.006 **	0.000 ***	0.000 ***	0.000 ***	0.000 ***
BSM1 vs. BSM3	0.015 *	0.030 *	0.030 *	0.000 ***	0.011 *	0.131 ^{ns}	0.014 *
The global *p*-value considering all samples	0.003 **	0.006 **	0.006 **	0.000 ***	0.001***	0.000***	0.000***

Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
BSM0 – control batch of beef snacks in the form of sticks, without the addition of maca; BSM1 – batch of beef snacks in the form of sticks with the addition of 1% maca; BSM3 – batch of beef snacks in the form of sticks with the addition of 3% maca.

The CIELab, Chroma, Hue and ΔE values of beef snacks with added maca are showed very highly significant differences ($p = 0.000$) between all product batches analyzed (The global p-value) (Table 3).

The brightness of the samples with added maca (BSM1 and BSM3) showed very highly significant decreases ($p < 0.001$) in mean values compared to the control lot (BSM0), where the maximum brightness value was obtained, being 46.80 ± 0.65 and decreasing to 44.13 ± 0.24 in the lot with 3% added maca (BSM3).

The intensity of the red color, identified by the parameter a*, showed significant decreases both according to the presence of the addition and the amount of it, between all the studied batches

(both as a whole and separately - two by two), very highly significant differences were observed ($p < 0.001$). The decrease in the CIEa* values from 18.03 ± 0.37 in batch BSM0 to 15.26 ± 0.27 in batch BSM3 reflects a reduction in the red color of the samples, most probably due to the presence of bioactive pigment compounds (e.g. polyphenols, flavonoids, carotenes), as we will see in Table 5.

The mean value of CIEb* (yellow-blue component) decreased very highly significant ($p = 0.000$) from 18.47 ± 0.48 in the BSM0 group to 17.42 ± 0.16 in the BSM1 group. However, the value of b* increased again in the BSM3 batch to 18.13 ± 0.14, the differences between this batch and the control batch (BSM0) being insignificant ($p = 0.211$), and the differences between the BSM3 and BSM1 batches were highly significant ($p = 0.008$). These results indicate that the addition of a low amount of maca, 1%, leads to a decrease in the yellow tones of the meat, followed by a concomitant increase with the increase in maca concentration in the meat samples.

The hue angle (hº) peaked in the control group (BSM0) at 44.31 ± 0.47, decreasing successively to 42.86 ± 0.28 in BSM1 and to 40.09 ± 0.44 in BSM3, the lowest value obtained by any of the three groups studied. Also, the differences between all the samples analyzed, both by groups of samples (BSM0 vs. BSM1, BSM0 vs. BSM3, BSM1 vs. BSM3) and in their totality (BSM0-BSM1-BSM3), were highly significant ($p = 0.000$).

The color saturation (C*) decreased very highly significant ($p = 0.000$) in the maca fortified samples (BSM1 and BSM3) compared to the control (BSM0), suggesting a decrease in the color intensity of the samples.

This decrease in color intensity may be due to the denaturation of meat-specific hemin pigments or to interactions between these and the specific pigments of *Lepidium meyenii* used in our study.

The results for ΔE (Total color difference) obtained mean values of 3.22 ± 0.62 for BSM0-BSM1 comparison and 3.89 ± 0.84 for BSM0-BSM1 comparison.

These values indicate that the differences are subjectively, not just objectively, visible and are sensory perceived by consumers (https://zschuessler.github.io).

Table 3. CIE L, a, b*, Chroma, Hue and ΔE colorimetric values of meat snacks with added maca (*Lepidium meyenii*)

Meat samples	Colour parameters					
	L*	a*	b*	Hue angle (hº)	Croma (C*)	ΔE
BSM0	46.80 ± 0.65	18.03 ± 0.37	18.47 ± 0.48	44.31 ± 0.47	25.81 ± 0.56	-
BSM1	44.42 ± 0.48	16.16 ± 0.10	17.42 ± 0.16	42.86 ± 0.28	23.76 ± 0.15	3.22 ± 0.62
BSM3	44.13 ± 0.24	15.26 ± 0.27	18.13 ± 0.14	40.09 ± 0.44	23.70 ± 0.24	3.89 ± 0.84
			p-value			
BSM0 vs BSM1	0.000***	0.000***	0.000***	0.000***	0.000***	-
BSM0 vs BSM3	0.000***	0.000***	0.211^{ns}	0.000***	0.000***	-
BSM1 vs BSM3	0.626^{ns}	0.001***	0.008**	0.000***	0.959^{ns}	0.235^{ns}
The global *p*-value considering all samples	0.000***	0.000***	0.000***	0.000***	0.000***	-

Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
BSM0 – control batch of beef snacks in the form of sticks, without the addition of maca; BSM1 – batch of beef snacks in the form of sticks with the addition of 1% maca; BSM3 – batch of beef snacks in the form of sticks with the addition of 3% maca.

The textural profile of the studied samples is presented in Table 4. The three analyzed samples can be observed in Table 4, and the influence of maca addition on the textural characteristics is evident. The addition of maca powder produced highly statistically significant changes in the parameters hardness, adhesiveness, elasticity and gumminess ($p < 0.001$), depending on the concentration used. The addition of maca powder (*Lepidium meyenii*) caused very highly significant ($p < 0.001$) changes in most textural parameters evaluated for beef snacks. The mean value of the textural parameter Hardness increased highly significantly ($p < 0.001$) with increasing maca concentration, from 26.35 ± 0.57 N in the control (BSM0) to 33.16 ± 1.52 N in the 1% maca (BSM1) and 36.66 ± 0.85 N in the 3% maca (BSM3). This increase indicates a progressive hardening of the product structure, most probably due to the decrease in product moisture content (Table 2) which is directly proportional to the increase in the concentration of added maca powder. Most likely, this

increase in the hardness of the samples is due to the high fiber content of the maca powders, which is more than 23% on a dry matter basis, as shown in the research of Chen et al. (2017).
Similarly, the adhesiveness showed highly significant differences ($p < 0.001$) between the studied batches, with an increase in mean values with increasing concentration of added maca powder, as in the case of the Hardness parameter, which may suggest a stickier texture of the batches with the added vegetable.
In terms of elasticity, a highly significant decrease was observed with increasing maca concentration (from 0.54 ± 0.02 in control to 0.39 ± 0.04 in BSM3). This result indicates a loss in the ability of the product to recover its original shape after deformation, which can be explained by the stiffening of the product network (explained by the increase in the dry substance in the sample by the addition of maca powder, Table 2).
Most probably maca is responsible for this loss of elasticity, because between BSM1 and BSM3, both batches with added maca powder, no significant differences are identified ($p > 0.05$), but between them and the control batch (BSM0) the differences are highly significant ($p < 0.001$).
Cohesiveness did not undergo statistically significant changes between the analyzed samples ($p > 0.05$), the values being relatively constant and in the range of 0.44-0.49, which indicates that the integrity of the products is similar within the samples. In contrast, maca powder caused highly significant differences in the gumminess parameter, which showed a highly significant increase ($p < 0.001$) in the BSM1 and BSM3 batches. The concentration of added maca also had a considerable effect, with significant differences ($p < 0.05$) between the 2 batches containing maca powder, but not as evident as between these and the control batch.
In contrast, the chewiness parameter did not show significant differences ($p = 0.244$) between all batches of maca-added beef snacks, although an increasing trend was observed between the BSM0 (6.25 ± 0.41 J) and BSM1 (6.92 ± 0.51 J) and BSM3 (6.88 ± 0.96 J) batches, respectively, but not statistically significant ($p > 0.05$).

Table 4. Texture profile of beef snacks with added maca powder

Meat samples	Colour parameters					
	Hardness (N)	Adhesiveness (mJ)	Elaticity	Cohesiveness	Gumminess (N)	Chewiness (J)
BSM0	26.35 ± 0.57	3.49 ± 0.23	0.54 ± 0.02	0.44 ± 0.02	11.64 ± 0.66	6.25 ± 0.41
BSM1	33.16 ± 1.52	4.15 ± 0.14	0.44 ± 0.02	0.47 ± 0.03	15.63 ± 0.71	6.92 ± 0.51
BSM3	36.66 ± 0.85	4.41 ± 0.07	0.39 ± 0.04	0.49 ± 0.05	17.81 ± 1.58	6.88 ± 0.96
			p-value			
BSM0 vs BSM1	0.000***	0.000***	0.000***	0.451^{ns}	0.000***	0.288^{ns}
BSM0 vs BSM3	0.000***	0.000***	0.000***	0.193^{ns}	0.000***	0.327^{ns}
BSM1 vs BSM3	0.001***	0.068^{ns}	0.019*	0.816^{ns}	0.019*	0.995^{ns}
The global *p*-value considering all samples	0.000***	0.000***	0.000***	0.208^{ns}	0.000***	0.244^{ns}

Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
BSM0 – control batch of beef snacks in the form of sticks, without the addition of maca; BSM1 – batch of beef snacks in the form of sticks with the addition of 1% maca; BSM3 – batch of beef snacks in the form of sticks with the addition of 3% maca.

The analysis of antioxidant compounds and antioxidant activity of beef snacks with maca powder (Table 5) highlights a strong influence of the addition of maca on the antioxidant profile of beef snacks. The analyses of antioxidant compounds and antioxidant activity were performed on both non-heat-treated and heat-treated samples, in order to evaluate possible changes (degradation or improvement) in antioxidant capacity induced by the heat treatment used. Maca, in the form used by us in the meat sticks (powder), showed a strong antioxidant activity, with inhibition values of 83.50% (DPPH) and 85.04% (ABTS), along with a flavonoid content of 0.98 mg CE/g DW and total polyphenols of 3.62 mg GAE/g DW.
The antioxidant activity values obtained by us recommend this product as having a high antioxidant potential, but the flavonoid and polyphenol content is not the highest, these results being specific for products obtained from roots, which are not exactly rich in these compounds, as shown in the study by Karimi et al. (2011), who conducted studies on several

types of *Labisa pumila* Benth, but also the research of Lee & Chang (2019), who, unlike us, performed methanol extractions of bioactive compounds from maca and obtained low results for polyphenols and flavonoids (0.17 ± 0.26 mg GAE/g DW and, respectively, 0.01 ± 0.17 mg CE/g DW), even lower than those obtained by us through ethanol extraction.

In meat samples without maca addition (BSM0N and BSM0T), the antioxidant activity was significantly lower (below 20% inhibition for both methods), and the content of phenolic compounds was also reduced (0.24-0.27 mg GAE/g DW). These values confirm that the beef base has a low native antioxidant activity.

The addition of maca powder led to increases in all analyzed parameters, but depending on the added concentration and the presence or absence of heat treatment. Samples with 3% non-heat-treated maca (BSM3N) recorded the highest values for antioxidant compounds: flavonoids (0.82 mg CE/g DW) and polyphenols (1.03 mg GAE/g DW), thus confirming the sensitivity of bioactive compounds to high temperatures. However, the antioxidant activity of this batch was lower than in the case of the sample with 3% addition of heat-treated maca (BSM3T), which presented inhibition values of 37.07 ± 0.13% (DPPH) and 39.13 ± 0.15% (ABTS), compared to the non-heat-treated sample with the same concentration of maca powder (BSM3N), whose oxidation inhibitory capacity was 33.70 ± 0.27% (DPPH) and 35.74 ± 0.43% (ABTS).

In contrast, heat treatment led to a slight decrease in the values for antioxidant compounds, observable in both flavonoid and polyphenol content for the BSM3T sample (0.76 mg CE/g DW and, respectively, 0.91 ± 0.02 mg GAE/g DW), but the effect was not drastic. This is supported by the literature, which indicates a certain stability of polyphenols to thermal processing of extraction of the compounds at 100ºC, since they do not have many hydroxyl substituents in their structure (Dai & Mumper, 2010). Our results show that they also resist after thermal processing of meat products at temperatures of 70-80ºC for 2-3h.

The content of β-carotene and total carotenoids was significantly higher ($p < 0.05$) in the non-heat-treated batches compared to the heat-treated batches. This difference suggests a possible partial degradation of these pigments following exposure to high temperatures. However, the content of lycopene increased significantly ($p < 0.05$) in the heat-treated samples compared to the non-heat-treated ones in all the batches studied, obtaining values of 0.07 ± 0.00 (BSM1T) and 0.12 ± 0.01 (BSM3T) in the heat-treated batches, compared to 0.06 ± 0.01 (BSM1N) and 0.11 ± 0.00 (BSM3N) in the non-heat-treated batches. Regarding the content of β-carotene, the non-heat-treated batch with 3% maca (BSM3N) recorded the highest concentration of it (0.34 mg/g d.w.), followed by BSM1N (0.23 mg/g d.w.). After applying the heat treatment, the average value decreased slightly in the BSM3T batch reaching 0.28 mg/g d.w., but increased to 0.25 mg/g d.w. in BSM1T. The decreasing trend in the BSM3T batch can be partially attributed to the thermal degradation and isomerization of β-carotene from its trans form (predominant in nature and stable) to the more unstable cis forms (Bernhardt & Schlich, 2006). The results between the batches with the addition of maca for total carotenoids, lycopene and β-carotene showed significant differences ($p < 0.05$). By comparing them with the values of these bioactive compounds in maca powder, the differences were very highly significant ($p < 0.001$).

Carotenoids are lipid-soluble compounds that are sensitive to light, oxygen, and temperature, and β-carotene and lycopene, in particular, are susceptible to isomerization and oxidation during heat treatments, leading to a decrease in stability and biological activity. Carotenoids are found in nature mainly in the more stable trans configuration, but small amounts of cis isomers are increasingly found. Because cis isomers have a different biological potency than their trans counterparts, these cis isomers have different biological activity compared to the trans form (e.g., they may be less or more effective as provitamin A) (Rodriguez-Amaya, 2001).

The antioxidant effect of maca powder was also observed by Półtorak et al. (2018), who evaluated a combination of plant ingredients (catuaba, galangal, roseroot, guarana and polyfloral honey), including maca root powder, added to the composition of some sausages, finding a significant increase in antioxidant activity (assessed by the DPPH method) during their storage.

Tabel 5. Antioxidant compounds content and antioxidant activity of beef snacks with added maca

Meat samples	Biochemical activity						
	Antioxidant Activity DPPH (% Inhibition)	Antioxidant Activity ABTS (% Inhibition)	Total flavonoids (mg CE/g DW)	Total polyphenols (mg GAE/g DW)	Total carotenoids (mg CT/g DW)	β-caroten (mg/g DW)	Lycopene (mg/g DW)
Maca	83.50 ± 0.31^{g}	85.04 ± 0.41^{g}	0.98 ± 0.00^{e}	3.62 ± 0.01^{e}	0.56 ± 0.00^{e}	0.48 ± 0.00^{e}	0.26 ± 0.00^{d}
BSM0N	16.77 ± 0.25^{a}	18.12 ± 0.21^{a}	0.29 ± 0.01^{a}	0.24 ± 0.02^{a}	-	-	-
BSM0T	17.75 ± 0.23^{b}	19.41 ± 0.11^{b}	0.29 ± 0.02^{a}	0.27 ± 0.02^{a}	-	-	-
BSM1N	29.51 ± 0.27^{c}	30.85 ± 0.48^{c}	0.58 ± 0.01^{b}	0.70 ± 0.04^{b}	0.32 ± 0.01^{b}	0.23 ± 0.01^{a}	0.06 ± 0.01^{a}
BSM1T	31.59 ± 0.28^{d}	33.80 ± 0.22^{d}	0.56 ± 0.01^{b}	0.67 ± 0.02^{b}	0.31 ± 0.01^{a}	0.25 ± 0.01^{b}	0.07 ± 0.00^{a}
BSM3N	33.70 ± 0.27^{e}	35.74 ± 0.43^{e}	0.82 ± 0.01^{d}	1.03 ± 0.02^{c}	0.46 ± 0.00^{d}	0.34 ± 0.01^{d}	0.11 ± 0.00^{b}
BSM3T	37.07 ± 0.13^{f}	39.13 ± 0.15^{f}	0.76 ± 0.02^{c}	0.91 ± 0.02^{d}	0.40 ± 0.01^{c}	0.28 ± 0.00^{c}	0.12 ± 0.01^{c}
	p-value						
The global *p*-value considering all samples	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***

Letters on the same column differ significantly.
Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
BSM0N – control batch of beef snacks in the form of sticks, without the addition of maca and not thermally treated; BSM0N – control batch of beef snacks in the form of sticks, without the addition of maca and thermally treated; BSM1N – batch of beef snacks in the form of sticks with the addition of 1% maca, not thermally treated; BSM1T – batch of beef snacks in the form of sticks with the addition of 1% maca, thermally treated; BSM3N – batch of beef snacks in the form of sticks with the addition of 3%, not thermally treated; BSM3T – batch of beef snacks in the form of sticks with the addition of 3%, thermally treated.

The sensory analysis of beef products with added maca is composed of 18 sensory characteristics and is presented in Table 6 and a graphical representation of consumer preferences is shown in Figure 1. Most of the sensory characteristics analyzed by the panelists in the panel analysis revealed that there were no significant differences ($p > 0.05$) among most of the sensory characteristics studied, these were as follows: Color uniformity; General aspect, Specific smell of meat, Plant odors, Unpleasant odor, Taste persistence, Taste balance, Tender, Juiciness, Desire to try the product again.

Very highly significant differences ($p = 0.000$) between samples were obtained for the sensory characteristics Foreign odors from meat products, Smoky taste and Foreign taste, and significant differences ($p < 0.05$) were identified within the characteristics Smoky odor, Specific meat product taste, Salty taste, Sweet taste and Firmness.

Smoky odor was diminished by the addition of maca in the samples, obtaining a score of 5.47 ± 0.74 in the BSM3 group and 6.00 ± 1.13 in the BSM1 group, both lower than the average score obtained by the control group (BSM0), 6.47 ± 0.99. Another sensory characteristic related to olfactory perception that was strongly influenced by the addition of maca is Foreign odors from meat products. As can be seen from the data in Table 6, the mean scores for the control batch (BSM0) and the batch with 1% maca powder addition (BSM1) are identical (1.00 ± 0.00), the major difference being observed only in the case of the batch with 3% added maca (BSM3), which obtained from the panelists the average score of 1.73 ± 0.70. From these results we can deduce that a low addition of maca powder does not influence the odor of the meat products studied, but higher concentrations (~3%) show a major influence on the olfactory perception of the odor of meat products, being perceived an uncharacteristic odor of the meat products. This result correlates with the characteristic Specific meat product taste which showed significant differences ($p = 0.040$) between the studied batches, its score decreasing concomitantly with the addition of maca, from 7.87 ± 1.19 in the BSM0 batch (no maca added) to 6.80 ± 1.01 in the BSM3 batch (batch with 3% maca added).

As well as smoky odor, smoky taste was also greatly affected by the addition of maca, decreasing its mean value in the control batch 5.33 ± 1.05 by about 2 points. This fact reveals that maca (*Lepidium meyenii*) has a pronounced

characteristic flavor that covers the flavor characteristic of the meat and those imprinted by the technological process of production. This taste, as identified by the tasters, is a sweet taste, which is uncharacteristic for such products (foreign tastes), but which is not considered unpleasant by consumers, since the characteristic Desire to try the product again did not change significantly between batches ($p > 0.05$), indicating that the products with added maca powder are similar in terms of enjoyment to classic products without added plant-based ingredients. Also, the addition of maca, as observed in the chemical analysis of the analyzed products (Table 2), showed a decrease in the mean value concomitant with the addition of maca, the differences between batches being significant ($p < 0.05$), but without negatively affecting the overall taste of the products. From this we can deduce that consumers do not perceive the decrease in saltiness as having a negative effect on the taste of meat products, as long as it is balanced.

Table 6. Sensory attributes evaluated in beef snacks enriched with maca (*Lepidium meyenii*): mean ± SD and statistical significance

Sensory attributes	Beef sticks sampples			*p*-value
	BSM0	BSM1	BSM3	
Colour uniformity	7.53 ± 0.99	7.53 ± 1.13	7.40 ± 1.06	0.924 (ns)
General aspect	7.73 ± 0.70	7.60 ± 0.83	7.80 ± 0.86	0.786 (ns)
Specific smell of meat	4.47 ± 1.19	4.27 ± 1.22	4.13 ± 1.13	0.740 (ns)
Smoky odor	6.47± 0.99	6.00 ± 1.13	5.47 ± 0.74	0.026 (*)
Plant odors	1.07 ± 0.26	1.20 ± 0.56	1.33 ± 0.62	0.359 (ns)
Foreign odors from meat products	1.00 ± 0.00	1.00 ± 0.00	1.73 ± 0.70	0.000 (***)
Unpleasant odor	1.13 ± 0.35	1.07 ± 0.26	1.07 ± 0.26	0.773 (ns)
Specific meat product taste	7.87 ± 1.19	7.60 ± 1.24	6.80 ± 1.01	0.040 (*)
Salty taste	5.87 ± 0.92	5.47 ± 0.74	5.00 ± 0.76	0.020 (*)
Smoky taste	5.33 ± 1.05	4.67 ± 0.62	3.53 ± 0.83	0.000 (***)
Sweet taste	2.27 ± 0.96	2.93 ± 1.28	3.47 ± 1.19	0.024 (*)
Foreign taste	1.07 ± 0.26	1.27 ± 0.59	2.60 ± 0.83	0.000 (***)
Taste persistence	4.87 ± 1.06	4.67 ± 0.82	4.40 ± 0.83	0.378 (ns)
Taste balance	6.33 ± 1.59	6.60 ± 1.24	7.27 ± 1.03	0.145 (ns)
Tender	6.07 ± 0.96	5.60 ± 1.18	5.13 ± 0.92	0.056 (ns)
Juiciness	4.47 ± 1.30	4.33 ± 1.29	3.93 ± 1.28	0.506 (ns)
Firmness	3.67 ± 1.05	4.13 ± 0.64	4.47 ± 0.74	0.038 (*)
Desire to try the product again	6.47 ± 1.73	6.53 ± 1.81	7.13 ± 1.41	0.485 (ns)

Levels of significance: ns – $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
BSM0 – control batch of beef snacks in the form of sticks, without the addition of maca; BSM1 – batch of beef snacks in the form of sticks with the addition of 1% maca; BSM3 – batch of beef snacks in the form of sticks with the addition of 3% maca.

The only texture-related sensory characteristic significantly affected ($p < 0.05$) by the addition of maca was Firmness, which recorded an increase in mean value directly proportional to the increase in maca powder content, reaching the maximum value in our study in the BSM3 lot 4.47 ± 0.74, which is 0.8 points higher than that of the control BSM0 lot, 3.67 ± 1.05. The External Preference Mapping (PrefMap) diagram presented in Figure 1 provides a two-dimensional graphical representation of consumer preferences for beef stick samples with the addition of yellow maca powder. This diagram is based on the mean scores of the experimental batches presented in Table 6.

In Figure 1, there are, as we can see, 6 areas of percentage preference, however, in the legend of the diagram, positioned on the right side of it, there are only 5 areas of percentage preference. As we can see on the diagram, there is an overlap of the red and yellow colors, which leads to an orange-brown color, the area on which all three batches of beef snacks enriched with maca (*Lepidium meyenii*) are positioned. This indicates that they fall within a preference range of 60-100%, which indicates that there are no major differences in their overall preference, at least not at a general level.

However, the placement of the samples in the same area and their proximity to the preference vectors of different consumer groups (clusters) indicate subtle variations in the preference of each of the segments.

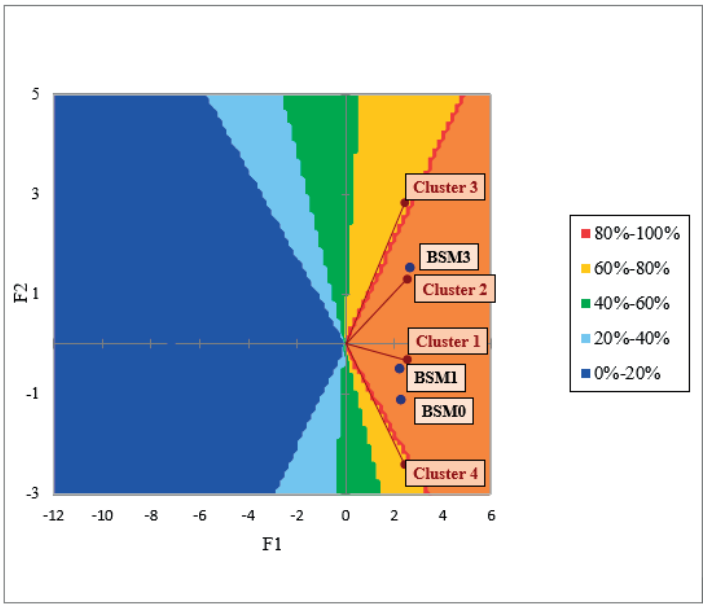


Figure 1. External Preferance Mappping (PrefMap)

(BSM0 – control batch of beef snacks in the form of sticks, without the addition of maca; BSM1 – batch of beef snacks in the form of sticks with the addition of 1% maca; BSM3 – batch of beef snacks in the form of sticks with the addition of 3% maca)

Results obtained from the sensory analysis show that the products are perceived similarly by consumers, without variations that affect the overall sensory perception. These results indicate that functional meat sticks with maca addition represent a viable option in the future of functional foods. The products were very well accepted by consumers, being appreciated not only for their balanced sensory profile, but also for their practical character, adapted to modern requirements for ready-to-eat foods, which do not require additional thermal preparation and can be consumed quickly, in various active life contexts.

CONCLUSIONS

The results obtained in this study highlight the potential of using maca powder (Lepidium meyenii) as a functional ingredient in the manufacture of meat products adapted to current and future consumer requirements.

The addition of maca in proportions of 1% and 3% contributed to the emergence of significant changes in the physicochemical, colorimetric, textural, antioxidant and sensory characteristics of the studied products, which in our case were represented by beef snacks (meat sticks).

The addition of maca powder to meat snacks significantly influenced the physicochemical parameters of the product, especially at a concentration of 3%. Very significant decreases in the content of fat, protein, collagen, moisture, salt and pH were observed, indicating an important compositional restructuring caused by the vegetable addition. These changes are explainable by the non-protein and acidic nature of maca powder.

Colorimetric analysis indicated a darker and less saturated appearance of maca products, changes that can be attributed to interactions between natural meat pigments and pigmentary (bioactive) compounds in maca.

Regarding texture, the addition of maca significantly influenced mechanical parameters, especially hardness, adhesiveness, elasticity and gumminess. These effects were dose-dependent, being more pronounced at 3%.

Antioxidant activity (DPPH, ABTS) and the content of bioactive compounds (flavonoids, polyphenols, carotenoids) increased proportionally with the level of added maca, but remained significantly lower compared to the values in the maca powder analyzed as such.

Sensory analysis highlighted that the addition of maca powder in proportions of 1% and 3%

influences certain sensory characteristics, especially those related to the taste and smell of the samples, firmness and the presence of foreign taste notes, without compromising the desire to consume this type of product again. The high scores for "Desire to try again" and the favorable positioning in the PrefMap analysis suggest a good acceptability of the maca-enriched products. Although the addition of maca attenuated the specific taste and smell of the meat products, it introduced a foreign sweet note that was not perceived as negative. The change in firmness (its increase) of the samples with the addition of maca was well tolerated and correlated with the maca concentration. In conclusion, maca powder can be successfully integrated into meat products, contributing to the improvement of the functional profile while maintaining, at the same time, the attractive sensory characteristics for consumers, and may represent a promising option for the development of functional meat products adapted to the requirements of the modern consumer.

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SUBSTITUTION OF SOYBEAN WITH CHICKPEA IN THE DEVELOPMENT OF A FUNCTIONAL POULTRY MEAT PRODUCT

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Abstract

Healthier meat foods are gaining in popularity and consumers are receptive to various plant-based protein sources, especially pulses. Pulses are highly versatile and a rich source of essential nutrients. They are also a source of high-quality protein, suitable for people of all ages and comparable to other protein-rich foods. Soya, which is the legume most commonly used in meat products, and its derivatives are among the most allergenic of a variety of foods that can cause negative reactions. Although chickpeas also belong to the same class as soya, the legumes, they are such a very common allergen. For this reason, we substituted soybean with chickpea in poultry meat products with heterogeneous and emulsion structure to observe the qualitative differences imprinted on the final products and the behaviour upon heat treatment. The obtained products were studied by physico-chemical analyses (pH, colour-texture, fat, protein, protein, moisture, salt content) and the obtained results were subjected to statistical tests to observe the existing differences.

Key words*: chicken meat product, experimental design, food formulation, meat processing, meat product development.*

INTRODUCTION

One of the main sources of vital nutrients for the human body is meat (Ciobanu et al., 2025). These nutrients, which serve a variety of purposes in the human body, include proteins, lipids, vitamins and minerals (Manoliu et al., 2023; Manoliu et al., 2024). These are essential for carrying out physiological and metabolic processes in a normal state, boosting immunity and fighting diseases such as malnutrition (Anchidin et al., 2024b; Ciobanu et al., 2023a), but also due to the fact that plant products and their derivatives do not provide these nutrients easily (Anchidin et al., 2024a). The benefits of meat are also present in meat products (Ciobanu et al., 2024). Due to its nutritional qualities, meat and meat products are recognized as necessary components of the human diet (Boișteanu et al., 2023). Nutritional and dietetic value are the main elements influencing consumer behavior when it comes to the consumption of meat and meat products (Ciobanu et al., 2023b).

Approximately 38% of global meat production was made up of chicken meat in 2021, with 135.4 million tons produced globally, an increase of 3.5% compared to 2019 (Tudorache et al., 2022), even if new dietary trends, predominantly Western societies, support the reduction or replacement of meat in the diet (Flocea et al., 2024).

Due to its numerous advantages, including its affordability, safety and influence on health through its protein intake, compatibility with processing and lack of religious restrictions on consumption, public interest in poultry meat production has increased (Tudorache et al., 2022), and the popularity of chicken-derived products has also led to this increase (Boișteanu et al., 2025).

Beyond financial limitations and the current energy crisis, one of the main problems of livestock farming and meat industry is its substantial contribution to air pollution (Popa et al., 2022). Under these circumstances, adding plant additives to animal products is a promising way to improve their nutritional and functional qualities while reducing the meat industry's environmental impact. In addition, the demand for natural, and especially organic, ingredients is growing as food and feed markets increasingly shift towards sustainable and natural products (Grigore et al., 2023), which

supports the incorporation of these plant-based substances into animal product formulations. Such incorporation could be achieved by partially replacing meat with legumes. The modification would facilitate addressing food security concerns and is in line with the advice of climate change experts (Lemken et al., 2019).

Legumes account for 27% of primary plant production worldwide (Smýkal et al., 2015). They are edible seeds belonging to the second largest family of seed plants, the *Leguminosae*, which has about 13,000 species and 600 genera. The word 'leguminous' comes from the Latin legere, meaning 'to gather', and refers to the manual collection of seeds. Pulses, derived from the latin *puls*, meaning "food made from flour", is another name for legumes. Soya is the world's most widely grown legume crop. Other legume crops include groundnut, dry bean, dry pea, chickpea, broad bean, field bean, lentil, maize, misc. bean, lupin and Bambara bean (Augustin et al., 2024).

Health risks associated with legume consumption are an increasingly important topic in both industrialized and developing countries. Numerous studies have shown that legume consumption can have both negative and positive health effects (Gupta et al., 2017). Despite being recognized as an important source of protein, soybeans and their derivatives are considered one of the main allergenic foods among the many foods that cause negative reactions (Ogawa et al., 2000). Although chickpeas also represent allergenic compounds, their allergenic potential and allergic reactions have a much lower prevalence than that of soybeans and other legumes, such as peanuts which have the highest allergenic potential among legumes (Cabanillas et al., 2018).

Chickpea, an increasingly important field crop, represents a sustainable and healthy plant component with added value for the food and nutraceutical industry (Augustin et al., 2024). Chickpeas are considered to have the richest nutritional content of all legumes on the market. Proteins and amino acids, simple carbohydrates, easily and poorly digestible polysaccharides (including dietary fiber), lipids, vitamins of different groups, and macro- and micro-nutrients are among the biogenic components found in this natural plant source, all of which are present in ideal amounts (Dzhaboeva et al., 2021). It is one of the most cost-effective sources of plant protein (25-30% protein) (Ghribi et al., 2018), being a cheap source for them and with very good bioavailability (Herrera & Gonzalez, 2021). This, as well as consumers' high desire for new products, allows companies to create new products that satisfy the market for goods with increased nutritional value (Herrera & Gonzalez, 2021; Anchidin et al., 2023a), given that consumers' food preferences are changing and are more complicated than manufacturers anticipate (Ciobanu et al., 2022; Pogurschi et al., 2018).

Chickpeas are large, salmon-white in color, and rich in protein (21.70-23.40%) and carbohydrates (41.10-47.42%). About 83.9% of all carbohydrates are composed of starch, making starch the main source of nutrition in chickpeas. Chickpea seeds are high in complex carbohydrates (low glycemic index), with high protein digestibility, rich in vitamins and minerals, and relatively free of antinutritional agents (Mittal et al., 2012). They have a fat content ranging from 3.10 to 5.67%, depending on the type, which is more than found in other legumes and some cereals, but lower than other oil legumes such as soybean and groundnut. Chickpea fat is composed of about 15% saturated fatty acids, 19% monosaturated fatty acids and 66% polyunsaturated fatty acids (Grasso et al., 2022).

The use of chickpeas in various forms (paste, flour, protein isolate) in meat products has been reported by various authors, with prevalence especially in the last 5 years, indicating an increased and topical interest in this type of addition in meat products: Verma et al., 1984; Sanjeewa et al., 2010; Ghribi et al., 2018; Wang et al., 2023a; Kasaiyan et al., 2023a; Wang et al., 2023b; Kasaiyan et al, 2023b; Mariod & Abd Elagdir, 2024. As part of contemporary food processing, various food quality enhancers are used (Zugravu et al., 2017) and chickpeas, as the literature has shown us so far, can be an excellent quality enhancer for meat products. Technological developments in the meat sector also enable the production of more sustainable and healthier food.

Our aim was to replace soy-based vegetable protein with chickpeas in meat products with heterogeneous and emulsion structure, because soy protein is a common allergen with a high prevalence in the population.

Chickpeas were chosen because they share many of the technological qualities of soybeans, as they belong to the same class of legumes, but chickpeas are not considered a common allergen.

We also wished to observe the qualitative differences imprinted by chickpeas on the finished products.

MATERIALS AND METHODS

The experimental batches were made from boneless chicken thigh purchased from S.C. Fermador S.R.L. in Iasi County, and the plant-based additives (soy and chickpeas) were purchased from S.C. Lidl Discount S.R.L. The seasoning mix used and the membranes used for the experimental batches were purchased from a local company in Iasi, S.C. Rocas FDS S.R.L. The raw materials and ingredients used in the batches studied are presented in Table 1 in percentage form.

Tabel 1. The experimental batches' composition and the thermal treatments that were used

Experimental batches	Raw materials and ingredients (%)						
	Chicken thigh	Chopped boiled soybeans	Chopped boiled chickpeas	Salt	Ground pepper	Sweet paprika	Garlic powder
MES	90	10	-	0.2	0.2	0.2	0.5
MMS							
CE		-	10				
CM							

MES - sample with heterogeneous structure and added soybean protein; MMS - sample with emulsion structure and chickpea protein; CE - sample with heterogeneous structure and added chickpea protein; CM - sample with emulsion structure and added chickpea protein.

Depending on the type of structure of the products (heterogeneous or emulsion) a differentiated heat treatment was used. The stages were drying, smoking and boiling. The temperature values and times of these stages are given in Table 2.

Table 2. Stages and parameters of the thermal regime applied to experimental plots

Structure type of the products	**Heat treatment**							
	Drying I		Smoking		Boiling		Drying II	
	T°C	t (min)	T°C	t (min)	T°C	t (min)	T°C	t (min)
Heterogeneous	55	20	80	25	78	30	80	5
Emulsion	60	30	70	20	78	30	80	5

The technological process consisted of coarse grinding (in the form of shreds) of deboned chicken pulp through the 0.8 mm diameter sieve of the volf for samples with heterogeneous structure. For samples with an emulsion structure, the coarse grind was further subjected to a fine grinding process by cutterization. These were mixed with the plant-based additives according to the experimental batch and the seasoning mix (Table 1). The obtained compositions were dosed into cellulose membranes (samples with heterogeneous structure) and collagen membranes (samples with emulsion structure) with a diameter of 45 mm, obtaining products with a length of about 20 cm. These were placed on racks and subjected to the heat treatment described in Table 2. After completion of the heat treatment, the samples were cooled, vacuum-packed and stored under refrigerated conditions (2-4°C).

The laboratory analyses carried out for the studied batches consisted of physico-chemical analysis, as follows: analysis of chemical parameters, pH measurement, color determination and texture evaluation.

Analysis of the crude chemical composition included determination of moisture, dry matter, protein, collagen, fat and salt content using the FoodCheck automated infrared analyzer (Bruins Instruments GmbH, KPM Analytics).

The physical measurements included: 1. Measuring the pH of the samples using the pH meter HI98163, with electrode FC2323 special for meat (S.C. Hanna Instruments S.R.L.) (Anchidin et al., 2023); 2. Measuring the color

indicators in the three-dimensional CIE color space which consisted of the parameters lightness (L*), redness (a*) and yellowness (b*) (Gucianu et al., 2023), using the Konica Minolta Chroma Meter CR-410 color analyzer (Konica Minolta, Inc.). Hue (H°) and Chroma (C*) parameters were calculated using formulas (1) and (2), based on the CIELAB parameters determined using the model of Anchidin et al. (2024a); 3. Textural profile analysis (TPA) was performed with the TA1+1K Plus texturometer (Ametek, Inc.) and the textural parameters hardness, elasticity, cohesiveness, gumminess, adhesiveness, chewiness were determined.

$$H° = tan^{-1} \times \frac{b^*}{a^*} \quad (1)$$

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (2)$$

In addition to the determinations described above a statistical test - Principal Component Analysis (PCA) which is a useful tool for assessment of correlations between the physical and chemical parameters studied contributing to the differentiation of the studied batches (Cozzolino et al., 2019; Boișteanu et al., 2024).

RESULTS AND DISCUSSIONS

The analyses carried out mainly focused on the influence of the type of structure (heterogeneous and emulsion), the influence of the type of vegetable additive (soy and chickpea) and the influence of these two influencing factors (type of structure*vegetable additive) on the overall quality of the samples studied (Table 3).

The moisture content of the analyzed samples showed significant differences ($p < 0.05$) in the interaction between the two tracked factors and for both types of vegetable addition. The heterogeneous structure type showed highly significant differences ($p < 0.001$), while the emulsion structure showed significant differences ($p < 0.05$). Mean moisture values were higher in the chickpea-added batches for both texture types. However, we can observe that the emulsified samples showed higher values for this parameter regardless of the vegetable addition, suggesting the major influence of the product structure on this parameter, but also the influence of the vegetable addition, which was not as significant. Our results are in slight contradiction with those obtained by Pennells et al. (2024) who looked at the functionality of some legume extruded ingredients. They showed that chopped soybean has a higher water-holding capacity than chickpeas. However, samples of unchopped chickpea extrudates showed a higher value of the same parameter than soybean within the same type of structure.

As with the moisture parameter, significant differences ($p < 0.05$) between the factors analyzed in this study (structure type*plant-based additive) were observed for the dry matter parameter. The type of plant-based additive showed highly significant differences ($p = 0.000$) in both the heterogeneous and emulsion structures. The heterogeneous structure type also showed the same trend as the vegetable additive type for this parameter, with highly significant differences being identified within the heterogeneous structure type ($p = 0.000$), but in the emulsion structure the differences were only significant ($p = 0.013$). The mean values of the studied batches for this parameter show that the addition of soybean leads to a slight increase in this parameter, especially in the heterogeneous structure, where this difference is more evident.

For the parameters protein, collagen, salt and pH no significant differences were found ($p > 0.05$), as can be seen in Table 3. In the case of protein the average values obtained were slightly lower with the addition of chickpeas, especially in the case of heterogeneous structure. As Messina (1999) also shows us, the protein content of soybean is almost double that of chickpeas, thus giving the products to which this chemical parameter is added a slightly higher value. However the differences were extremely small within the products, where soybean showed a protein value 0.08% higher for the heterogeneous structure and 0.02% higher for the emulsified products. The collagen content did not show differences, as it is not found in the plant-based

products, nor did they influence this parameter in any way. Also, the salt content did not show significant differences between the analyzed batches ($p > 0.05$), regardless of the type of addition. This is due to the extremely low sodium content in legumes, as observed by Meiners et al. (1976). Even if they showed that the sodium content is in much lower amount in the soybean, being at trace level, we observed this aspect in our study only relatively, since the highest average value was obtained by the product with heterogeneous structure and soybean addition (1.53 ± 0.67%), but also the lowest value for the emulsified product with the same addition (1.48 ± 0.08%). In the case of the chickpea-added samples, its content was constant in both types of structures (1.50 ± 0.07%).

Table 3. Mean values, standard deviations and significance levels of the physicochemical parameters for the analyzed samples

Analyzed parameters	Structure type	Plant-based additive	Mean ± standard deviation of mean	Significance for the plant-based additives (soy - chickpea) (p-value)	Significance for the type of structure, heterogeneous-emulsion (p-value)	Significance between type of structure*plant-based additive (p-value)
Humidity (%)	Heterogeneous	Soy Protein	70.91 ± 0.12	0.000***	0.000***	0.035*
		Chickpea Protein	71.30 ± 0.12	0.000***		
	Emulsion	Soy Protein	72.16 ± 0.05	0.000***	0.013*	
		Chickpea Protein	72.34 ± 0.09	0.000****		
Dry Matter (%)	Heterogeneous	Soy Protein	29.09 ± 0.12	0.000***	0.000***	0.035*
		Chickpea Protein	28.70 ± 0.12	0.000***		
	Emulsion	Soy Protein	27.84 ± 0.05	0.000***	0.013*	
		Chickpea Protein	27.66 ± 0.09	0.000***		
Protein (%)	Heterogeneous	Soy Protein	21.08 ± 0.08	0.710^{ns}	0.150^{ns}	0.434^{ns}
		Chickpea Protein	21.00 ± 0.07	0.461^{ns}		
	Emulsion	Soy Protein	21.06 ± 0.11	0.710^{ns}	0.710^{ns}	
		Chickpea Protein	21.04 ± 0.05	0.461^{ns}		
Collagen (%)	Heterogeneous	Soy Protein	19.08 ± 0.13	1.000^{ns}	0.430^{ns}	0.457^{ns}
		Chickpea Protein	19.14 ± 0.11	0.297^{ns}		
	Emulsion	Soy Protein	19.08 ± 0.13	1.000^{ns}	0.791^{ns}	
		Chickpea Protein	19.06 ± 0.09	0.297^{ns}		
Fat (%)	Heterogeneous	Soy Protein	6.32 ± 0.04	0.000***	0.000***	0.457^{ns}
		Chickpea Protein	5.96 ± 0.11	0.000***		
	Emulsion	Soy Protein	5.16 ± 0.15	0.002***	0.002**	
		Chickpea Protein	4.88 ± 0.13	0.002***		
Salt (%)	Heterogeneous	Soy Protein	1.53 ± 0.67	0.297^{ns}	0.527^{ns}	0.457^{ns}
		Chickpea Protein	1.50 ± 0.07	1.000^{ns}		
	Emulsion	Soy Protein	1.48 ± 0.08	0.297^{ns}	0.672^{ns}	
		Chickpea Protein	1.50 ± 0.07	1.000^{ns}		
pH	Heterogeneous	Soy Protein	6.42 ± 0.11	0.251^{ns}	0.303^{ns}	0.490^{ns}
		Chickpea Protein	6.49 ± 0.02	0.849^{ns}		
	Emulsion	Soy Protein	6.50 ± 0.10	0.251^{ns}	0.949^{ns}	
		Chickpea Protein	6.50 ± 0.12	0.849^{ns}		

The mean ± standard deviation is used to display the values (n = 5). Statistical significance is indicated as follows: $^{ns}p \geq 0.05$ – not significant; $*p < 0.05$ – statistically significant; $**p < 0.01$ – highly significant; $***p < 0.001$ – extremely significant.

For the pH parameter, the highest mean values, irrespective of the type of additive, were found in the emulsified structure, being identical for both types of additives (6.50), and within the heterogeneous structure, the chickpea sample showed a slightly higher value of this parameter (6.49 ± 0.02%) compared to the soy containing sample (6.42 ± 0.11%). From this we can deduce that the structure had a slight influence on this parameter, but extremely low and insignificant.

In terms of crude fat content, both the type of structure and the type of vegetable addition showed highly significant ($p < 0.001$) and, for the type of emulsifying structure, highly significant ($p < 0.01$) effects on the crude fat content, but the interaction of all these variables was insignificant ($p > 0.05$). The chickpea-added samples obtained slightly lower mean values in this analysis, which may be correlated with the higher moisture content in the chickpea-added samples. Our results are in agreement with the literature showing higher fat content in soybeans (Perkins, 1995) compared to chickpea (Iqbal et al., 2006). However, the feed matrix (heterogeneous or emulsion) also plays an obvious role in the case of this parameter, as it can be observed that the fat content in the soy emulsified sample is lower than in the chickpea heterogeneous sample, but higher than in the chickpea emulsified sample. These results show both the influence of the vegetable addition on the fat content, but also the influence of the structure, which in this case is significant but not major.

The instrumental color analysis of the experimental plots is shown in Table 4 and includes the parameters L*, a*, b*, h° and C*.

The analyzed samples showed extremely significant differences ($p < 0.001$) in the correlation between type of structure*plant-based additive for most parameters (CIE L*, CIE a* and H°), highly significant differences ($p < 0.01$) were observed only within the CIE b* parameter, and in the case of C* the differences were insignificant ($p = 0.388$). The brightness of the samples (L*) showed higher average results in the case of emulsified samples, this fact being due to the higher homogeneity of the samples and the higher moisture content of the emulsified samples (Table 3). Research conducted on chicken meat color by Qiao et al. (2001) showed that chicken meat samples with a lighter color (higher brightness) also showed a significantly higher moisture content compared to chicken meat samples with a lower brightness. Also, within the same type of structure, extremely significant differences ($p = 0.001$) were identified depending on the type of plant-based addition, with higher average values being observed for products with chickpea addition compared to samples with soy addition, this fact being correlated with the higher water retention capacity of samples with chickpea addition, regardless of structure. Even though the plant-based addition had a great influence on the colorimetric parameter L*, the type of structure showed extremely significant differences between the studied batches ($p = 0.001$), regardless of the type of plant-based addition. This trend is also observed in the case of the chemical parameter humidity, where the emulsified samples recorded higher values in the emulsified samples than in the heterogeneous ones.

The CIE a* parameter (referring to the red-green component), compared to the CIE L* colorimetric parameter values, showed an inversely proportional trend of increase or decrease. This trend of decrease in the a* parameter following the increase in the L* parameter was also observed in a previous study conducted by Anchidin et al. (2023c) on pork.

Thus, samples with heterogeneous structure presented higher average values than those with emulsified structure, with extremely significant differences being recorded between the two types of structures studied ($p < 0.001$). Also, the type of plant-based addition presented extremely significant differences in the case of all four batches studied ($p < 0.001$).

The highest values for the CIE parameter a*, 11.07±0.27 and 11.60 ± 0.09, were observed in samples with heterogeneous structure for soybean addition and chickpea addition, respectively. In case of emulsion structure the average results obtained were 10.20 ± 0.10 for soybean and 9.80 ± 0.12 for chickpea.

As can be seen, even though in the case of heterogeneous structure, the chickpea addition showed a higher value than soybean and the tendency, according to the cited work and our results is to decrease the degree of red color, in

this case it increases proportional to the increase in the brightness value, being the only case of this kind in our study. The emulsified sample with added chickpeas, on the other hand, which presented the highest brightness value (67.41 ± 0.26), also obtained the lowest value in the colorimetric parameter a* (9.80 ± 0.12), results that are in line with the literature.

Table 4. Mean values, standard deviations and significance levels of the colorimetric parameters for the analyzed samples

Analyzed parameters	Structure type	Plant-based additive	Mean ± standard deviation of mean	Significance for the plant-based additives (soy - chickpea) (*p*-value)	Significance for the type of structure, heterogeneous-emulsion (*p*-value)	Significance between type of structure*plant-based additive (*p*-value)
CIE L*	Heterogeneous	Soy Protein	61.64 ± 0.27	0.000***	0.000***	0.000***
		Chickpea Protein	62.20 ± 0.13	0.000***		
	Emulsion	Soy Protein	65.68 ± 0.09	0.000***	0.000***	
		Chickpea Protein	67.41 ± 0.26	0.000***		
CIE a*	Heterogeneous	Soy Protein	11.07 ± 0.27	0.000***	0.000***	0.000***
		Chickpea Protein	11.60 ± 0.09	0.000***		
	Emulsion	Soy Protein	10.20 ± 0.10	0.000***	0.001***	
		Chickpea Protein	9.80 ± 0.12	0.000***		
CIE b*	Heterogeneous	Soy Protein	15.66 ± 0.31	0.204^{ns}	0.378^{ns}	0.004**
		Chickpea Protein	15.56 ± 0.10	0.003**		
	Emulsion	Soy Protein	15.52 ± 0.10	0.204^{ns}	0.001***	
		Chickpea Protein	15.94 ± 0.09	0.003**		
h°	Heterogeneous	Soy Protein	35.25 ± 1.11	0.000***	0.002**	0.000***
		Chickpea Protein	36.71 ± 0.38	0.000***		
	Emulsion	Soy Protein	33.33 ± 0.38	0.000***	0.000***	
		Chickpea Protein	31.57 ± 0.20	0.000***		
C*	Heterogeneous	Soy Protein	19.18 ± 0.17	0.000***	0.006**	0.388^{ns}
		Chickpea Protein	19.41 ± 0.03	0.000***		
	Emulsion	Soy Protein	18.57 ± 0.07	0.000***	0.069^{ns}	
		Chickpea Protein	18.71 ± 0.13	0.000***		

CIE L* - luminosity of the samples; CIE a* - red-green component; CIE b* - yellow-blue component; H° - hue angle indicating the direction of color in the chromatic space; C* - chroma, indicates color saturation values. The mean ± standard deviation is used to display the values (n = 5). Statistical significance is indicated as follows: $^{ns}p \geq 0.05$ – not significant; $*p < 0.05$ – statistically significant; $**p < 0.01$ – highly significant; $***p < 0.001$ – extremely significant.

As for the colorimetric parameter CIE b*, it was not significantly influenced by the addition of soy, its influence being insignificant ($p > 0.05$). Despite these results, the addition of chickpea protein had a very significant influence on this parameter ($p < 0.01$). The samples with heterogeneous structure showed insignificant differences ($p = 0.378$) within this parameter, while differences between samples with emulsified structure were highly significant ($p = 0.001$). Statistical differences concerning the correlation between structure type and plant-based addition were highly significant ($p = 0.004$) for this parameter, this

was less significant than those for the parameters CIE L* and CIE a*. The average results obtained for parameter b* did not show a certain linearity or order due to the type of plant addition or the type of structure and can be considered slightly chaotic, without a clear trend.

The hue angle (h°), as well as the b* parameter, showed the same slightly chaotic trend of the average results, depending on the type of plant-based addition. However, a trend is observed in the case of the type of structure of the samples: samples with heterogeneous structure recorded higher average values (35.25 ± 1.11 for soybean and 36.71 ± 0.38 for chickpea) than samples with emulsion structure (33.33 ± 0.38 for soybean and 31.57 ± 0.20 for chickpea) which indicates a very slightly more reddish color of the emulsified samples, compared to the heterogeneous ones, which have a slightly more yellow tint, as we can see on the colorimetric wheel presented by Cantrell et al. (2010). The types of plant-based additives added in all samples (soybean/chickpea) show highly significant differences ($p < 0.001$), as well as in the interaction between the type of palnt-based additive and the product structure. The differences between the type of structure for the analyzed samples are highly significant ($p = 0.002$) while the emulsified structure shows highly statistically significant differences ($p = 0.000$). Chroma (C*), which represents the perceived color saturation, showed higher average values in the case of samples with heterogeneous structure than in the case of those with emulsion structure. Also, they were lower in the case of samples with added soy protein (19.18 ± 0.17 – heterogeneous sample and 18.57 ± 0.07 – emulsified sample) than in the case of samples with added chickpea protein (19.41 ± 0.03 – heterogeneous sample and 18.71 ± 0.13 – emulsified sample). The differences recorded by the type of plant-based addition within all the samples studied are extremely significant ($p < 0.001$). Regarding the type of structure, the differences observed are insignificant in terms of emulsified samples ($p = 0.069$) and very significant in heterogeneous samples ($p = 0.006$). The correlation type of plant-based addition*type of structure for the C* parameter showed that the differences are not significant between the samples studied ($p > 0.05$).

Texture profile analysis for the 4 batches analyzed is presented in Table 5 and consists of the following textural parameters: hardness, adhesiveness, elasticity, cohesiveness, gumminess, chewiness.

The hardness of the samples showed no significant influence ($p > 0.05$) on the type of plant-based additive added or the structure of the samples. The highest average value of this parameter was identified in the case of the batch with heterogeneous structure and soybean addition (12.88 ± 1.55 N), and the lowest value was identified within the same type of structure, but with the addition of chickpea (11.22 ± 0.88 N). Despite these results, in the case of the emulsion structure, the highest hardness value was identified in the sample with the addition of chickpea protein (11.93 ± 2.67 N), the emulsified sample with the plant-based additive of soybean obtaining the value of 11.22 ± 0.88 N, which is very close to the value of the heterogeneous sample with the addition of chickpea.

Regarding the adhesiveness of the samples, higher average values were observed in the case of meat samples with the addition of soy vegetable protein, both with heterogeneous structure (86.76 ± 6.57 mJ) and with emulsion structure (62.03 ± 7.41 mJ), this fact being also observable by the extremely significant differences of the samples with this type of plant-based addition ($p < 0.001$). The samples with the addition of chickpea protein showed lower average values, the lowest being identified within the batch with emulsified structure (47.81 ± 4.31 mJ). The addition of chickpea led to the lack of statistically significant differences in adhesiveness in the batches containing it ($p > 0.05$). However, the type of structure showed extremely significant statistical influences in the case of heterogeneous structure ($p < 0.001$) and very significant in the case of emulsified structure ($p < 0.01$). The interaction between the type of structure and the addition of plant-based protein reported very significant differences ($p = 0.004$). The same interaction in the case of the textural parameter elasticity did not present statistical differences ($p = 0.538$). This was more influenced by the type of structure, being

identified similar average results in the case of the emulsified structure with the addition of soy (0.60 ± 0.03) and with the addition of chickpeas (0.54 ± 0.03), the statistical differences within this structure for elasticity being insignificant (p = 0.066). For the heterogeneous structure, the elasticity parameter showed significant differences between the two analyzed samples (p = 0.011), these reporting lower results than within the emulsified structure and with greater differences between the average values obtained (0.46 ± 0.05 – sample with added soy and 0.37 ± 0.08 – sample with added chickpea).

Table 5. Mean values, standard deviations and significance levels of the texture profile analysis (TPA) of the tested samples

Analyzed parameters	Structure type	Plant-based Additive	Mean ± standard deviation of mean	Significance for the plant-based additives (soy – chickpea) (*p*-value)	Significance for the type of structure, heterogeneous-emulsion (*p*-value)	Significance between type of structure*plant-based additive (*p*-value)
Hardness (N)	Heterogeneous	Soy Protein	12.88 ± 1.55	0.167^{ns}	0.141^{ns}	0.156^{ns}
		Chickpea Protein	11.22 ± 0.88	0.519^{ns}		
	Emulsion	Soy Protein	11.34 ± 1.00	0.167^{ns}	0.586^{ns}	
		Chickpea Protein	11.93 ± 2.67	0.519^{ns}		
Adhesiveness (mJ)	Heterogeneous	Soy Protein	86.76 ± 6.57	0.000***	0.000***	0.004**
		Chickpea Protein	53.17 ± 6.87	0.204^{ns}		
	Emulsion	Soy Protein	62.03 ± 7.41	0.000***	0.003**	
		Chickpea Protein	47.81 ± 4.31	0.204^{ns}		
Elaticity	Heterogeneous	Soy Protein	0.46 ± 0.05	0.000***	0.011*	0.538^{ns}
		Chickpea Protein	0.37 ± 0.08	0.000***		
	Emulsion	Soy Protein	0.60 ± 0.03	0.000***	0.066^{ns}	
		Chickpea Protein	0.54 ± 0.03	0.000***		
Cohesiveness	Heterogeneous	Soy Protein	0.66 ± 0.03	0.000***	0.001***	0.759^{ns}
		Chickpea Protein	0.55 ± 0.05	0.001***		
	Emulsion	Soy Protein	0.78 ± 0.04	0.000***	0.000***	
		Chickpea Protein	0.65 ± 0.05	0.001***		
Gumminess (N)	Heterogeneous	Soy Protein	8.50 ± 1.02	0.734^{ns}	0.001***	0.265^{ns}
		Chickpea Protein	6.18 ± 0.94	0.065^{ns}		
	Emulsion	Soy Protein	8.80 ± 0.58	0.734^{ns}	0.312^{ns}	
		Chickpea Protein	7.89 ± 2.29	0.065^{ns}		
Chewiness (J)	Heterogeneous	Soy Protein	3.90 ± 0.81	0.015*	0.005**	0.421^{ns}
		Chickpea Protein	2.24 ± 0.44	0.001***		
	Emulsion	Soy Protein	5.29 ± 0.60	0.015*	0.055^{ns}	
		Chickpea Protein	4.23 ± 1.20	0.001***		

The mean ± standard deviation is used to display the values (n = 5). Statistical significance is indicated as follows: $^{ns}p \geq 0.05$ – not significant; $*p < 0.05$ – statistically significant; $**p < 0.01$ – highly significant; $***p < 0.001$ – extremely significant.

The type of plant-based addition, in terms of elasticity and cohesiveness of the samples, had an extremely significant influence on all the analyzed samples ($p < 0.001$). In the case of cohesiveness, the same extremely significant differences were also observed in the case of the two types of structures targeted in our study ($p < 0.001$). However, the correlation between the type of structure and the plant-based addition in the analyzed samples was insignificant ($p > 0.05$). For the same parameter, the samples with soybean addition presented higher average values (0.66 ± 0.03 – heterogeneous structure; 0.78 ± 0.04 – emulsified structure) than those with chickpea (0.55 ± 0.05 – heterogeneous structure; 0.65 ± 0.05 – emulsified structure), but the structure also had an important influence, with higher values being observed for the emulsified samples. A higher cohesiveness of some meat emulsions with plant-based addition of soy, but in the form of soybean oil, was observed in the study of Shao & Tang (2014) who motivated this by increasing the resistance of the protein gel. Our study shows that not only soybean oil has this property, but soybean itself. As the results show us, chickpea does not have the power to form a very strong cohesiveness, but the results are satisfactory and the products have an appropriate cohesiveness.

Gumminess and chewiness both showed higher mean values in the case of samples with soybean addition, especially in the case of heterogeneous structure, as can be seen in Table 5. Gumminess and chewiness showed extremely significant differences ($p < 0.001$) and, respectively, significant ($p < 0.01$) in the case of heterogeneous structure and insignificant in the case of emulsion structure ($p > 0.05$). Also, the interaction between the type of structure of the samples and the added plant-based ingredients did not show significant differences between the analyzed samples ($p > 0.05$). The same result was obtained in the case of the type of plant-based addition in the case of the textural parameter gumminess, where the differences were insignificant regardless of the plant-based addition ($p > 0.05$). However, the same results were not obtained in the case of chewiness, where the results were highly significant for the plant-based addition of chickpea protein ($p < 0.001$) and significant for the addition of soy protein ($p < 0.05$).

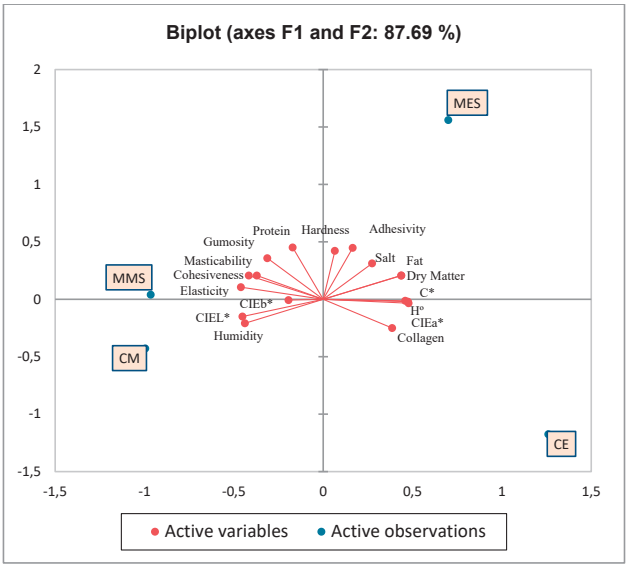


Figure 1. Relationships between the physico-chemical attributes studied by using the Principal Component Analysis (PCA). MES - sample with heterogeneous structure and added soybean; MMS - sample with emulsion structure and soybean; CE - sample with heterogeneous structure and added chickpea; CM - sample with emulsion structure and added chickpea

Figure 1 presents the correlations between all the physical and chemical parameters analyzed in this study to observe the positive or negative influence between them. The PCA biplot covers 87.69% of the total variability. The sample with emulsified structure and soy protein addition (MES) is correlated with the attributes hardness, adhesiveness, fat and dry matter, mainly, highlighting results with higher values for these physicochemical characteristics. The emulsified sample with soy addition (MMS) is characterized, in particular, by better cohesiveness, elasticity, chewiness and gumminess. In contrast, the protein content between these two samples is very similar being positioned relatively median between the 2 analyzed samples.

The sample with chickpea addition and heterogeneous structure is characterized by a higher content of collagen, dry matter, fat, but also higher values of the colorimetric parameters CIE a*, hº and C*. In contrast, the sample with chickpea addition and emulsified structure correlates very positively with the MMS sample and is characterized by physicochemical characteristics such as: humidity, high brightness (CIE L*), yellow color (CIE b*), elasticity, cohesiveness, chewiness and gumminess.

The results obtained in the case of the PCA biplot also show the superiority of the textural characteristics of the samples with the addition of soybean (MES and MMS), compared to those with the addition of chickpea (CM and CE). However, the sample with the addition of chickpea and with a texture closer to that imprinted by the addition of soy in meat products is the emulsified sample (CM), which correlates positively with the textural characteristics elasticity, chewiness, cohesiveness and, to a lesser extent, gumminess. Also, a positive correlation can be observed between the samples with the same type of structure, these presenting many common characteristics, especially in the case of the samples with emulsified structure, regardless of the type of plant-based addition (MMS and CM). They present strong correlations between the analyzed physico-chemical characteristics. These results show us that the type of structure has the greatest influence on the physico-chemical characteristics of the analyzed samples. These results are especially valid for samples with emulsified structure. Although in samples with heterogeneous structure, regardless of the type of plant-based addition, the physicochemical characteristics are positively correlated, they are not as close and similar as in the case of the emulsified structure type. The type of structure shows positive and weakly positive correlations in the case of samples with soybean addition (MES and MMS). In contrast, the samples with chickpea addition (CE and CM) show weakly positive correlations or they are neutral. Strong negative correlations are observed between samples with different structure types and different plant-based addition, such as CM - MES and MMS - CE which show strong antagonism.

CONCLUSIONS

The replacement of soybean by chickpea in chicken meat products with heterogeneous structure and emulsion structure was aimed to follow the effects of this modification on some of the most important physico-chemical parameters of interest for meat products. Our research focused both on the differences imparted by the plant-based addition and those imparted by the type of product structure.

As a result of our quantitative research and especially with the help of correlations realized by Principal Component Analysis (PCA), it was found that both the type of structure and the plant-based addition have an important influence on the physico-chemical parameters studied. Samples with the same type of structure, even if they have different types of additions, show similar results in most of the analyses.

The samples with emulsion structure showed very significant similarities highlighting the fact that the influence on the classic product (the one with added soybean) of the chickpea addition is not major and can be easily substituted by it. As for the samples analyzed with heterogeneous structure, it cannot be said that the homogeneity of the results between the two is as high as in the case of the emulsified ones, but the differences are not major, with a few exceptions.

The physical results revealed significant differences in the color of the samples as a function of both the added mixture and the structure of the samples. On the other hand, the texture of the samples did not show differences for the correlation between these factors, except for the adhesiveness where differences were observed especially between the two types of additions.

The chemical parameters showed that the addition of chickpea had a very positive influence on the water holding capacity, as evidenced by the obvious increase in moisture content in the lots containing this type of addition. The protein content, although lower in chickpeas than in soybeans, did not affect the protein content in the samples analyzed except by a very slight decrease of this constituent to a negligible extent. A very positive effect of added chickpeas was observed in the decrease in the fat content of the samples, which is in line with current industry trends.

From the results we can conclude that the addition of chickpeas instead of soybean is a promising option for the industry that does not affect to a large extent the classical (soy-added) products, but even leads to quality improvements.

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APPLICATION OF RED ALGAE (*Palmaria palmata*) IN BRINE-INJECTED PORK LOIN: IMPACTS ON PRODUCT QUALITY PARAMETERS

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Abstract

The trend in the meat industry towards healthy products, coupled with growing concerns about the nutritional and ethical aspects of meat consumption, has led to an increased interest in new approaches to formulate meat products that contain non-meat ingredients. The use of natural marine-derived ingredients as functional additives in meat products is due to their ability to enrich the nutritional value and provide antioxidant properties. The purpose of this study is to evaluate the functional potential of red algae (Palmaria palmata) as a natural additive in smoked meat products. This study focused on the effects of red algae extract in brine injection solutions in the formulation of 3 batches of pork loin (injected with 10% [w/w]): 1 control (with a standard brine) and 2 experimental batches (brine with 1% and 2% extract of Palmaria palmata). Batches were heat-treated and evaluated in terms of physicochemical composition, color parameters, texture profile and sensory perception. The experimental batches showed an improvement in protein content, a reduction in fat and salt content and color changes by increasing lightness (L) parameter and reduction of red color intensity (a*).*

Key words*: brine injection, meat product formulation, meat quality, Palmaria palmata.*

INTRODUCTION

Meat is a major source of protein in many people's diets, and people are highly adaptable to meat consumption. In addition, meat is a significant source of micro- and macronutrients in addition to protein, such as essential fatty acids, vitamins, minerals and bioactive compounds, some of which are only available or have higher bioavailability in animal products (Pogurschi et al., 2018; Leroy and Cofnas, 2020; Boișteanu et al., 2023). However, in recent decades, meat production and consumption have come under scrutiny due to major concerns related to the livestock sector, sustainability and ethical aspects of meat production, along with health issues related to meat consumption (Font-i-Furnols, 2023).

In general, additives are used in all branches of the food-producing industry with the clear goals of improving product quality (Zugravu et al., 2017; Flocea et al., 2024). Sodium chloride (NaCl) and phosphates are commonly used additives in the manufacture of meat products that have both preservative and sensory enhancement roles (Ciobanu et al., 2024b). NaCl acts synergistically with phosphates in the extraction and solubilization of myofibrillar proteins, which are responsible for emulsification of water-holding capacity and fat retention (Glorieux et al., 2017). Phosphates can also increase oxidative and microbiological stability as well as improve tenderness and juiciness of meat products (Powell et al., 2019). High levels of NaCl and phosphate negatively affect the nutritional quality of meat products, with an impact on consumer health. Excessive consumption of NaCl, the main source of sodium in meat products, can cause numerous health problems, such as high blood pressure, cardiovascular disease, kidney disease, stroke, gastritis, metabolic syndrome and obesity (Cappuccio et al., 2019; Gonçalves et al., 2016; Taylor et al., 2018).

To counteract this negative impact due to the additives increasingly used in the meat industry, new ingredients from natural sources are being sought with the aim of obtaining healthier protein products (Anchidin et al., 2023; Manoliu et al., 2024). An example in this regard is the integration of functional

ingredients, such as vegetable additives (spinach, red lentils) that significantly influence the sensory and physico-chemical profile of meat products (Gucianu et al., 2023; Ciobanu et al., 2025; Boișteanu et al., 2025).

Red algae (*Palmaria palmata*) represent a valuable and functional alternative due to their rich content in proteins, essential amino acids, vitamins and bioactive compounds. They can provide products with antioxidant and shelf-life prolonging properties, thus contributing to improve the nutritional and functional value of meat preparations (Wang et al., 2021).

Therefore, this study aims to evaluate the impact of injecting brine solutions with two variants of red seaweed powder addition (1% and 2%) into pork chops on quality characteristics. The injected products, both the experimental variants and the control sample (injected with a standard brine), are subjected to the smoking heat treatment and evaluated from a physico-chemical and sensory point of view.

MATERIALS AND METHODS

The three formulations of smoked pork loin were obtained in the meat processing workshop of the Iasi University of Life Sciences, as shown in Table 1.

The pork loin was purchased from a local producer in Iasi county, SAGROD, and the seaweed was purchased from ALGAMAR, certified organic red algae (*Palmaria palmata*) in dehydrated form. To prepare the brine, the seaweeds were hydrated in water for 2 hours, then finely ground and added to the brine.

Table 1. Formulations and heat treatment of experimental batches for pork loin injected with different concentrations of red algae

Experimental batches	Injection level	Brine composition	Red algae content in brine	Thermal treatment		
				Air drying (°C/min)	Smoking (°C/min)	Hot air cooking (°C/min)
CC0%	10%	Water + salt	0%	65/30	72/30	86/50
RA1%		Water + salt + red algae	1%			
RA2%		Water + salt + red algae	2%			

CC0% - Control pork loin batch; RA1% – Pork loin with 1% red algae in brine; RA2% – Pork loin with 2% red algae in brine

After the finished products were obtained, they were qualitatively evaluated in the Meat and Meat Products Technology Laboratories and the Sensory Analysis Laboratory.

The proximate chemical composition was determined by a rapid method using the NIR FoodCheck analyzer (Bruins Instruments, OmegAnalyzer).

Determination of colour parameters (L*, a* and b*) was carried out using the Chroma Meter CR-410 (Konica Minolta Inc., Japan), set with D65 light source, 2° observation angle. Based on the obtained values, the following colour parameters were calculated, according to the formulas presented in a previous study (Ciobanu et al., 2024a):

- the colour intensity: $C^*=\sqrt{[(a^*)^2+(b^*)^2]}$;
- hue angle: $H^*=tan^{-1}(\frac{b^*}{a^*})$;
- total colour difference: $\Delta E=\sqrt{[(L^*_s-L^*_{con})^2+(a^*_s-a^*_{con})^2+(b^*_s-b^*_{con})^2]}$.

The determination of the pH value of the products was performed using a direct insertion pH meter (HI98163, Hanna Instruments Romania).

The determination of the textural parameters of shear (force and mechanical work) and compression (hardness, cohesivity, elasticity, gumminess, chewability and adhesiveness) was carried out using a TA1Plus texturometer (Lloyd Instruments, AMETEK, UK), equipped with a 500N force cell. The sample preparation stage for the Warner-Bratzler shear test involved the formation of 18 x 10 mm samples, and 30 mm high sections for the compression test.

The sensory evaluation session involved the organization of a sensory evaluation session in the sensory laboratory under controlled lighting and temperature conditions using a panel of 50 semi-trained evaluators (15 males and 35 females), aged between 20 and 22 years, all of whom were regular pork consumers. In order to familiarize the participants with the product

concept, they underwent a training session that included a presentation of the finished product and explanation of the hypothesis.

Given the added algae, affective methods of analysis were used and samples were prepared by uniform portioning, coded with three randomly chosen codes. The questionnaire was implemented in digital format (using google forms platform to centralize the answers) and the scale used was from 1 to 9, where 1 - dislike at all and 9 - like extremely.

The obtained results were statistically evaluated and are expressed as mean values (± standard deviation) and the experimental batches were compared with the control using analysis of variance (ANOVA) followed by Tukey's test at 5% significance level ($p < 0.05$), using IBM SPSS Statistics v.21.

RESULTS AND DISCUSSIONS

The chemical composition of the brine-injected pork loin formulations is presented in Table 2.

In the present study, the inclusion of red algae *(Palmaria palmata)* caused a significant effect ($p < 0.001$) on all analysed chemical parameters when compared to the control sample (CC0%). More precisely, the samples with 1% (RA1%) and 2% (RA2%) red algae dissolved into the brine solution displayed higher humidities, with the highest value observed for RA2% (74.74 ± 0.11%), indicating improved water holding capacity. Likewise, the content of dry matter, fat, and salt were decreased significantly with the increasing incorporation level of red algae, which can be attributed to the higher moisture of the investigated samples treated with the marine algae.

These results are consistent with the results of previous studies on algae addition in meat products, such as the one conducted by Muraguri (2018) who previously found that the addition of seaweed powder (*Eucheuma denticulatum)* into chicken sausage resulted in a better water-holding capacity and lower fat content. Likewise, Hentati et al. (2018) reported that supplementation of burgers with 1% algae (*J. adhaerens* and *C. compressa*) would lead to reduced fat content with an enhancement of technological properties, such as swelling capacity, water and oil holding capacity.

Regarding the protein content, a slight but statistically significant increase was observed for the RA2% sample (21.94 ± 0.05%), which could be due to the protein content from the red algae extract. These results are in agreement with the studies of Idowu (2024) and Cox and Abu-Ghannam (2013) who pointed out that marine algae could have a potential to be used as a source of protein to enrich food matrices. The collagen content, with was reported to the protein content, was comparable in all samples, meanwhile, which indicated that the muscle protein structural integrity was not significantly affected by the addition of algae. Siladji et al. (2024) also reported an increase in protein content in caseless sausages with the addition of three different types of algae (White *C. vulgaris,* sea spaghetti and wakame).

Table 2. Analysis of chemical composition of brine-injected pork loin formulations

Analyzed parameter	Sample			*p-value*
	CC0%	RA1%	RA2%	
Humidity (%)	72.28 ± 0.43 a	73.86 ± 0.36 b	74.74 ± 0.11 c	< 0.001
Dry Matter (%)	27.72 ± 0.43 c	26.14 ± 0.36 b	25.26 ± 0.11 a	< 0.001
Fat (%)	4.40 ± 0.07 c	2.64 ± 0.11 b	1.84 ± 0.17 a	< 0.001
Protein (%)	21.36 ± 0.05 a	21.78 ± 0.04 b	21.94 ± 0.05 c	< 0.001
Colagen*	19.70 ± 0.07 a	20.14 ± 0.09 b	20.30 ± 0.07 c	< 0.001
Salt (%)	1.94 ± 0.11 b	1.54 ± 0.05 a	1.44 ± 0.13 a	< 0.001

CC0% - Control pork loin batch; RA1% – Pork loin with 1% red algae in brine; RA2% – Pork loin with 2% red algae in brine. Values are given as means ± Standard deviation from five repeted determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test. *Colagen content is expressed as % from the total protein content.

The colorimetric parameters of brine-injected pork loin formulations are presented in Table 3. The addition of red algae significantly affected ($p < 0.05$) the color parameters L*, a*, b* and C* of the experimental batches, compared to the control (CC0%).

The values for lightness (L*) increased with the percentage of algae added in the brine solution,

with the highest L* value found in RA2% (69.31 ± 0.70), indicating a visual brightness of the pork loin samples. This increase in lightness (L*) can be positively correlated with the moisture content and the dilution of myoglobin concentration, that causes the characteristic red color to become paler and the meat to appear brighter.

Regarding the green-red coordonate (a*), a significant decrease in values was detected with algae addition, dropping from 14.23 ± 0.46 in the control sample to 9.54 ± 1.18 in RA1% and 8.15 ± 0.35 in RA2%. This indicates a reduction in the red intensity of the samples, making the surface appear paler and less vivid in red tones.

The b* (yellow-blue coordonate) showed a significant decrease following the addition of red algae into the brine solution. Color saturation (Chroma, C*) also decreased progressively with increasing algae content, reflecting a reduction in overall color intensity. Although the hue angle (h*) increased slightly with the addition of algae – indicating a slight shift toward a more yellowish hue – the difference was not statistically significant ($p = 0.066$). Total color difference (ΔE) values revealed that both RA1% and RA2% caused perceptible changes in color, with ΔE values of 8.22 ± 0.52 for RA1% and 4.08 ± 2.42 for RA2% compared to the control.

These findings align with those reported by Vilar et al. (2020), who observed that the incorporation of edible algae in frankfurters decreased redness (a*) and yellowness (b*) values in comparison to the control sample. Gullón et al. (2021) state that the color changes occur due to the Maillard reaction between the algae and pork meat, resulting in the formation of dark compounds, and also to the specific colouration of the red algae, that has a darker appearance.

Overall, adding red algae into the brine solution to be injected into pork loins resulted in lighter, less red products with lower color intensity – features that must be cautiously evaluated with respect to consumer expectations, as visual appearance is a decisive factor in the acceptability of meat products. Studies have showed that the effectiveness of traditional functional compounds, such as protein derivatives and starch, in improving the functional properties of meat emulsions (Ianițchi et al., 2023), as well as the influence of technological parameters on tenderness and color (Pătrașcu et al., 2021).

Table 3. Colorimetric parameters of brine-injected pork loin formulations

Analyzed parameter	L*(D65)	a*(D65)	b*(D65)	C*	h*	ΔE	Generated Hex colour
CC0%	65.12 ± 0.47a	14.23 ± 0.46c	14.07 ± 0.34b	20.02 ± 0.36c	44.70 ± 1.28a	-	
RA1%	66.77 ± 3.57ab	9.54 ± 1.18b	8.28 ± 0.27a	12.65 ± 0.97b	41.13 ± 3.36a	8.22 ±0.52	
RA2%	69.31 ± 0.70b	8.15 ± 0.35a	8.05 ± 0.52a	11.47 ± 0.42a	44.63 ± 2.30a	4.08 ±2.42	
p-value	0.027	< 0.001	< 0.001	< 0.001	0.066	-	-

CC0% - Control pork loin batch; RA1% – Pork loin with 1% red algae in brine; RA2% – Pork loin with 2% red algae in brine. L*(D65) – lightness (ranging from 0 = black to 100 = white); a*(D65) – red-green component (positive values = red tones, negative values = green); b*(D65) – yellow-blue component (positive values = yellow tones, negative values = blue); C* – chroma (reepresents the color saturation or intensity, higher values indicate more vivid colors); h* – hue angle (expresses the dominant color tone); ΔE – total color difference. Values are given as means ± Standard deviation from five repeted determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

The addition of red algae into brine solutions used for the injection of pork loin significantly influenced the meat's textural characteristics, as determined by the ANOVA statistical test applied on the results presented in Table 4 (Warner-Bratzler shear force measurements and Texture Profile Analysis - TPA). A dose-dependent enhancement in tenderness was observed, with the RA2% treatment (2% red algae) exhibiting the most pronounced effects.

The Warner-Bratzler shear force decreased significantly from 18.61 N/cm^2 in control

(CC0%) to 14.25 N/cm^2 in RA1% and 10.85N/cm^2 in RA2%, respectively ($p = 0.001$). Similarly, the work of shear was also significantly reduced with the increasing red algae concentration ($p = 0.004$). These decreases indicate that red algae components, such as carrageenan, may disrupt muscle fiber integrity and enhance moisture retention, leading to improved tenderness. This result aligns with the observations made by Cofrades et al. (2008), who reported that the addition of hydrocolloids from seaweeds favored the water and fat binding in low-salt meat emulsions, with subsequent alterations in product texture

A significant decrease in hardness was also observed, from 33.14 N in the control to 24.26 N in RA1% and 19.69 N in RA2% ($p < 0.001$). Gumminess and chewiness also decreased with increased doses of red algae ($p = 0.004$ and $p < 0.001$, respectively), reflecting a more tender and palatable texture. Those changes are in accordance with the study by López-López et al. (2009) where the incorporation of seaweed in functional frankfurters decreased hardness and the textural parameters.

While cohesiveness did not show statistically significant differences ($p = 0.124$), a trend towards increased values in the RA2% treatment suggests potential improvements in the structural integrity and resilience of the meat matrix. Adhesiveness significantly decreased from 0.78 in the control to 0.50 in RA2% ($p < 0.001$), indicating a low adhesive surface texture. Such decrease could be related to the water binding capacity of red algae constituents, which can modify the surface properties of meat products. Similar findings were reported by Kim et al. (2014), who observed decreased adhesiveness in cooked sausages formulated with red and green glassworts (*Salicornia herbacea* L.).

Table 4. Warner Bratzler shear forces and textural parameters of brine-injected pork loin formulations

Analyzed parameter	Sample			*p-value*
	CC0%	RA1%	RA2%	
Shear force (N/cm^2)	18.61 ± 1.53 b	14.25 ± 3.38 a	10.85 ± 1.32 a	0.001
Work of shear (mJ)	448.55 ± 6.08 b	345.93 ± 4.34 a	293.23 ± 3.80 a	0.004
Hardness (N)	33.14 ± 2.95 b	24.26 ± 4.37 a	19.69 ± 2.82 a	< 0.001
Cohesiveness (N)	0.21 ± 0.05 a	0.25 ± 0.08 a	0.44 ± 0.28 a	0.124
Elasticity	1.86 ± 0.15 b	1.43 ± 0.34 a	1.09 ± 0.13 a	0.001
Gumminess (N)	4.35 ± 0.85 b	3.35 ± 0.43 a	2.80 ± 0.28 a	0.004
Chewiness (N)	8.01 ± 1.22 b	4.85 ± 1.67 a	3.06 ± 0.59 a	< 0.001
Adhesiveness	0.78 ± 0.08 b	0.57 ± 0.09 a	0.50 ±0.05 a	< 0.001

CC0% - Control pork loin batch; RA1% – Pork loin with 1% red algae in brine; RA2% – Pork loin with 2% red algae in brine. Values are given as means ± Standard deviation from five repeted determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

The hedonic sensory evaluation of the three variants of injected pork loin - the control batch (CC0%) and the two experimental batches with the addition of 1% (RA1%) and 2% (RA2%) red algae - showed good overall consumer acceptability for all samples, with average scores above 7 on a 9 - point scale (Table 5). This observation suggests that regardless of the addition of red algae, the product was perceived positively and was found to be palatable by the panel of assessors.

The control sample (CC0%) received the highest scores for appearance (7.86 ± 0.42) and section appearance (7.54 ± 0.95), indicating a consumer preference for traditional visual characteristics. The addition of red algae seems to have negatively influenced the section appearance (scores of 6.43 and 6.29), with the results indicating statistically significant differences between the control and the RA1% and RA2% samples. This effect can be attributed to the presence of the algal insert resulting from the injection, perceived as atypical compared to the control.

In terms of colour, the scores were relatively similar, however the mean value for RA2% (6.75 ± 1.15) was significantly lower than for RA1% (6.82 ± 0.95) and control (7.65 ± 0.88), suggesting that the higher algal addition may impart a less familiar or attractive colour hue.

For flavor and taste, the addition of seaweed extract had a beneficial effect on the evaluators' perception. Both RA1% and RA2% samples scored significantly higher than the control, with RA2% reaching 8.22 ± 0.85 (flavor) and 8.43 ± 0.88 (taste), in contrast to the control

values of 7.58 and 7.60, respectively. These data suggest that seaweed may provide umami flavor notes or additional flavor complexity that may be well received by consumers.

Texture showed higher scores in the algae extract samples, especially in the RA2% (7.37 ± 1.20) compared to the control (6.89 ± 1.16), suggesting an effective tenderization of the meat possibly influenced by the enzymatic effect of the algae extract or colloidal compounds present in the algae.

Overall acceptability was high for all three batches, but the highest was obtained by RA2% (8.17 ± 0.82), with significant differences from the control. This result reflects the fact that, despite some reservations in terms of visual appearance, consumers perceived positively the overall sensory experience of products with added red algae, especially due to taste and flavor.

According to the results, the addition of red seaweed was hedonically accepted by consumers without significantly exceeding the control sample scores. This trend is also supported by the literature, which points out that high levels of addition may compromise attributes such as flavor and appearance, even though the nutritional benefits remain evident. In addition, maximum sensory acceptability appears to be achieved at low concentrations (≤2.5%) of seaweed extract, which is also consistent with the findings of author Mohammed (2024).

Table 5. Sensory analysis of brine-injected pork loin formulations

Sensory attribute	Samples		
	CC0%	RA1%	RA2%
Appearance	7.86 ± 0.42^{a}	7.61 ± 1.23^{ab}	7.33 ± 1.14^{b}
Section appearance	7.54 ± 0.95^{a}	6.43 ± 1.05^{b}	6.29 ± 0.32^{b}
Color	7.65 ± 0.88^{b}	6.82 ± 0.95^{a}	6.75 ± 1.15^{ab}
Flavor	7.58 ± 1.28^{b}	8.17 ± 0.74^{a}	8.22 ± 0.85^{a}
Taste	7.60 ± 0.85^{b}	8.37 ± 0.97^{a}	8.43 ± 0.88^{a}
Texture	6.89 ± 1.16^{a}	7.54 ± 1.39^{ab}	7.37 ± 1.20^{b}
Overall acceptability	7.73 ± 0.75^{a}	7.92 ± 0.81^{ab}	8.17 ± 0.82^{b}

CC0% - Control pork loin batch; RA1% – Pork loin with 1% red algae in brine; RA2% – Pork loin with 2% red algae in brine. Values are given as means ± Standard deviation. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

CONCLUSIONS

This study investigated the impact of incurporating red algae into brine solutions for pork loin injection, aiming to develop a reformulated meat product with improved textural properties and potential functional benefits derived from marine-derived polysaccharides.

The study results showed that the inclusion of red algae in the brine formula improved the nutritional value of pork loin by increasing water and protein content, but decreased fat and salt content of pork loins and promoted the development of healthier meat products. These findings are in accordance with the increasing amount of data that support algae as a source of functional ingredients that is capable not only of providing structure and texture but also of delivering nutritionally enhanced and sustainable foods to the consumer.

From a technological perspective, the incorporation of red algae into the brine solutions significantly influenced the colorimetric properties of pork loin. Lightness (L) increased with the addition of red algae, while the red-green coordinate (a) and yellow-blue coordinate (b*) decreased progressively, indicating a shift toward less saturated red and yellow tones. Consequently, chroma (C*) decreased, and the hue angle (h*) showed a slight but non-significant reduction. These changes suggest a perceptible but modest alteration in visual appearance. However, the total colour difference (ΔE) remained below the threshold generally considered perceptible to consumers, particularly for the RA2% sample (ΔE = 4.08 ± 2.36), implying that the reformulated products retained an acceptable visual quality.

Therefore, in terms of texture modifications, the addition of red algae into pork loin brine solutions significantly enhanced textural properties, particularly at a 2% concentration. Improvements in tenderness, gumminess,

chewiness, and overall palatability suggest that red algae can serve as a functional ingredient in meat processing, offering potential benefits for product quality and consumer satisfaction. These findings are corroborated by existing literature on seaweed applications in meat products, highlighting the value of red algae as a natural texturizing agent.

The results confirm the viability of using red algae in the formulation of injected pork loin from the consumer's perspective. While some visual attributes may be slightly affected, the benefits on flavor, taste and texture may offset this, leading to a high overall acceptance of the product. Communication strategies on the functional benefits of seaweed could also improve visual perception among consumers.

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ENHANCING PORK TENDERLOIN QUALITY PARAMETERS THROUGH SOY PROTEIN ISOLATE BRINE-INJECTION

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Abstract

This study aimed to evaluate the influence of soy protein isolate on the quality parameters of pork tenderloin to obtain a product with improved characteristics. Four experimental batches were prepared: a control batch (without soy protein isolate) and three batches injected with brine containing 1%, 2%, and 3% soy protein isolate, based on the weight of the meat. The injection process was carried out under controlled conditions. All batches were analysed from a physico-chemical perspective, and sensory evaluation was conducted by a panel of 50 semi-trained evaluators to assess product acceptability. The addition of soy protein isolate had a positive effect on water retention capacity and texture improvement. Treated batches showed increased values for parameters associated with mechanical resistance and a decrease in cohesion. CIELab colorimetric analysis indicated a significant increase in the treated samples` L (lightness) values, while the a* (redness) component decreased. Overall, the results demonstrated that injecting pork tenderloin with up to 3% soy protein isolate leads to qualitative changes, with sensory acceptability being more favorable for the 1% and 2% treated batches.*

Key words*: brine injection, plant-based protein additives, pork tenderloin optimization.*

INTRODUCTION

Protein of animal origin plays an important role in human consumption, representing an indicator of the economic status of the population, and consumers are showing increasing interest in the health and functionality of the food they consume (Anchidin et al., 2023). Meat is the raw material that provides essential nutrients such as proteins, fats, vitamins, minerals and essential amino acids, providing an overall energy supply to the human body (Chiurciu et al., 2024).

Meat processing is associated with a health risk, an aspect that can be improved by reducing the components used in the meat meal, with studies highlighting that their reduction may lead to technological limitations (Ciobanu et al., 2024a).

From a consumption point of view, pork and pork preparations are the most consumed types of meat globally, and their improvement is important to ensure consumer health and satisfaction (Covaciu et al., 2024).

Modern meat processing technologies utilize the injection of brines into the meat material, followed by mechanical treatments such as tenderizing, thus the salting process has a diffusive-osmotic character and its acceleration is achieved by the combined application of physical and chemical methods (Dementieva et al., 2021; Ciobotaru et al., 2024). For obtaining meat preparations, the most optimal solutions are the injection of multicomponent brines, which in addition to the main additions include functional elements such as soy protein isolate (Anchidin et al., 2024).

The aim of the study was to evaluate the impact of brine injection with added soy protein isolate in pork tenderloin. The main objectives of the study were to make the product and to analyse qualitative parameters, with focus on the analysis of textural and colour parameters on heat treated products.

MATERIALS AND METHODS

In order to achieve the proposed objectives, it was proposed to develop three types of brine-injected pork tenderloin with the addition of 1, 2 and 3% soy protein isolate (SPI). The pork tenderloin was purchased from a local producer

in Iasi County, SAGROD S.A, and the isolate was purchased from Redis Nutrition, without flavors and sweeteners.

For the realization of the experimental batches, the procedure described by Flocea et al. (2024) for the preparation of pork tenderloin (*Psoas major muscle*) was followed, these being carried out in the micro production workshop of USV Iași.

The preparation of the brine was carried out in four variants, the first one being used for the control batch, and the others, in which soy protein isolate was added in proportions of 1, 2 and 3% in the basic brine with a reduced salt concentration, according to Table 1. Each batch was injected in a proportion of 10% by mass of meat.

Tabel 1. Formulation of brine solution

Brine components	CB	B1	B2	B3
M.U.	%			
Water	97	96	95	94
Salt	2.5			
Soy protein isolate	0	1	2	3

CB – control brine, B1 – brine 1% addition, B2 – brine 2% addition, B3 – brine 3% addition.

The heat treatment stages were performed according to the protocol described in the previous study (Ciobotaru et al., 2024) applied for the smoked chop assortment, using the smoking cell. The process included the technological steps of lifting, smoking, cooking, maintaining the same time and temperature conditions (Table 2).

Tabel 2. Heat treatment parameters applied to the product

Heat treatment	Time (min)	Temperature (°C)
Air drying	30	65
Smoking	30	72
Hot air cooking	50	86

After obtaining the finished product, the experimental batches were subjected to physico-chemical analysis, carried out in the laboratory of meat and meat preparations technology of USV Iași. The determination of the parameters related to the chemical composition was carried out as described by (Ciobanu et al., 2024b), using the FoodCheck infrared spectrophotometer, determining the parameters moisture, fat, protein, collagen and salt content.

The colour was determined using Chroma Meter CR-410 colorimeter (Konica Minolta Inc., Japan) in the CIELAB scale according to the procedure performed by Manoliu et al. (2023).

The TA Plus Texturometer (AMETEK/Lloyd Instruments) with a maximum measuring capacity of 500 N was used to determine textural parameters.

For the determination of textural parameters, the samples were prepared in cylindrical form of 2 x 2 cm hexagonal dimensions using a standard device. The determinations were performed using the Warner-Bratzler test for shear force evaluation (shear force - N and mechanical shear work - mJ) and compression test for texture profile analysis (TPA), for the analysis of hardness, elasticity, cohesivity and adhesion parameters. The samples were analyzed within 24 hours after processing and maintained at 0-4°C.

The instrument was operated and data were recorded using NEXYGENPlus software, which allows precise control of the test parameters. The software also facilitates the extraction of textural parameters based on predefined formulas and integration zones.

The sensory evaluation of the experimental batches was carried out in the sensory analysis laboratory under controlled lighting and isolation conditions and was performed with the help of 50 semi-trained evaluators. The sex ratio of assessors was 15 males and 45 females, aged between 20-22 years and consumers of pork products. To understand the product, the evaluators went through a training process which consisted of presentation of the finished product, explanation of the specific attributes of the meat products as well as the attributes targeted in the session.

For the actual conduct of the evaluation session, the samples were uniformly portioned and randomly coded with a 3-digit code and served at optimal temperature. The online Google forms platform was used to create the questionnaire, which was structured by each attribute (appearance, odour, taste, aroma and texture) using the intensity scale from 1 to 9 where 1 represents extremely unpleasant and 9 extremely pleasant.

To analyse consumer preferences for pork samples injected with soy protein isolate (SPI),

an affective sensory evaluation was performed, followed by a multivariate analysis using the Preference Mapping (PrefMap) method. The aim of this method was to correlate the preference scores given by consumers with the sensory positioning of the samples in principal component space.

Four pork tenderloin samples were analysed, coded as follows:

243 - control lot, no addition of SPI (pork tenderloin injected with basic brine only);

141 - pork tenderloin injected with 1% soy protein isolate;

351 - pork tenderloin injected with 2% soy protein isolate;

611 - pork tenderloin injected with 3% soy protein isolate.

All these analyses were performed using XLSTAT, an add-in software for Microsoft Office Excel (Trial Version 2025, Addinsoft, Paris, France. The results are expressed as mean ± standard deviation.

RESULTS AND DISCUSSIONS

The results obtained for the chemical composition of the batches with soy protein isolate and the control batches are shown in Table 3. Following the addition of soy protein isolate it can be observed that the moisture content increased progressively, being directly proportional to the amount of isolate added. Astfe, the values ranged from the control batch (76.96%) to 74.96% for the 3% injected batch. The differences for this parameter are significant (p = 0.030), which indicates that the addition of soy protein isolate increases the moisturizing capacity of the meat, as it retains water better. Similar studies indicate a similar trend, such as that of Ha et al. (2019), who observed moisture increases in meat preparations injected with vegetable protein.

The values obtained for dry matter ranged from 20.88% for the control batch to 25.05% for the 3% batch with a significant difference between the results (p = 0.030). The results obtained indicate an indirect relationship with moisture, as higher water content dilutes the solid components. The injection of soy protein isolate in the experimental batches increased the value of crude protein content, obtaining values ranging from 21.24% (1% injection) - 21.74 (3% injection) compared to the control batch (20.88%), an increase due to a better retention capcaity of the protein fractions in meat Zhang X. et al. (2024).

Collagen recorded values ranged from 19.08% for the control group, and the highest value was recorded for the 3% injected group of 19.72%. Analyzing the results, these can be correlated with a lipid dilution resulting from higher water retention and increased total mass by injection. In the study by Do Santos Junior et al. (2020), they demonstrate that the addition of soy protein isolate to pork results in improved protein networks and textural properties.

Table 3. Mean (± standard deviation) of physico-chemical parameters for the samples of injected pork tenderloins

Samples	Statistical estimators	Physico-chemical parameters (%)						
		Humidity	Dry Matter	Protein	Colagen*	Fat	Salt	pH
LC0%	$\bar{X}$±sx	72.96 ± 0.07	27.04 ± 0.07	20.88 ± 0.10	19.08 ± 0.10	4.46 ± 0.05	1.54 ± 0.05	6.13 ± 0.01
LISP1%		73.22 ± 0.06	26.78 ± 0.06	21.24 ± 0.07	19.26 ± 0.05	4.18 ± 0.05	1.32 ± 0.04	6.08 ± 0.02
LISP2%		73.98 ± 0.06	26.02 ± 0.06	21.48 ± 0.04	19.46 ± 0.05	3.18 ± 0.12	1.24 ± 0.05	5.95 ± 0.02
LISP3%		74.96 ± 0.09	25.04 ± 0.09	21.74 ± 0.04	19.72 ± 0.06	2.04 ± 0.11	1.18 ± 0.04	5.72 ± 0.04
	p value	0.030	0.030	0.029	0.030	0.017	0.051	0.030

Values are given as means ± Standard deviation from five repeated determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test. *Collagen content is expressed as % from the total protein content.

The addition of soy protein isolate decreased the fat content in the treated batches, with significant differences (p = 0.017). The control batch recorded values of 4.46% while the experimental batches recorded values ranging from 4.18% (1% batch), 3.18% (2% batch) and 2.04% (3% batch). The decrease can be explained by the dilution of lipids but also by the injection process which contributed to the partial dispersion of lipids during processing. A constant decrease in the salt content is observed, being the highest in the control batch (1.54%) reaching the minimum average value (1.18%-3% batch). This indicates that this type of addition influences the salt content by increasing the moisture content of the samples, leading to a significant reduction in the salt content and is in line with current consumer requirements. The pH values followed a decreasing trend registering 6.13 for the control

batch, while the experimental batches recorded values ranging from 6.08 to 5.72. The decrease in pH may be attributed to the slightly acidic character of the soy protein isolate as well as the injection process. The color parameters (L*, a*, b*, C*, h°) are represented in Table 4.

Chromatic analysis performed on the injected experimental batches (LC0%, LISP1%, LISP2%, LISP3%) shows significant changes in color parameters depending on the treatment applied, reflecting the compositional and structural impact of soy protein isolate on the optical characteristics of the product.

Table 4. Mean (± standard deviation) of colour parameters for the samples of injected pork tenderloins

Parameters	Batches				p-value
	LC0%	LISP1%	LISP2%	LISP3%	
L*(D65)	69.322±1.352	71.912±0.519	72.352±0.582	71.02±0.687	0.034
a*(D65)	12.81±0.244	8.394±0.318	9.606±0.299	7.900±0.470	0.003
b*(D65)	8.394±0.395	9.200±0.501	8.310±0.676	8.448±0.281	0.032
C*	15.319±0.196	12.138±0.333	11.817±0.612	12.797±0.079	0.010
h°	33.230±1.567	49.337±2.829	44.655±2.037	41.334±1.800	0.072

L*(D65) – lightness (ranging from 0 = black to 100 = white); a*(D65) – red-green component (positive values = red tones, negative values = green); b*(D65) – yellow-blue component (positive values = yellow tones, negative values = blue); C* – chroma (reepresents the color saturation or intensity, higher values indicate more vivid colors); h* – hue angle (expresses the dominant color tone); Values are given as means ± Standard deviation from five repeted determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

The values of the lightness parameter (L*) were significantly higher for the batches injected with soy protein isolate ($p = 0.034$), indicating a tendency for the meat color to open up after the treatments. As regards the intensity of the a* (red-green) coordinate, a significant reduction ($p = 0.003$) was observed for the experimental batches, with the most marked decrease being recorded by batch LISP3 (7.900±0.470). For coordinate b* (blue-yellow), the LISP1 sample showed a slight enhancement of yellow nutation compared to the control batch.

The C* parameter was significantly lower in the treated batches, especially in LISP2% (11.817) and LISP1% (12.138) compared to LC0% (15.319). This indicates a decrease in color saturation, i.e. a duller, less vivid color. The result is consistent with the decreases observed in a* and suggests that SPI treatments may induce a 'masking' effect on the natural pigmentation of the meat.

Although the nunaune angle (h°) showed an increase for injected batches, the differences were not significant ($p = 0.072$).

The analysis of the textural parameters of the injected muscle, presented in Table 5, shows significant differences between the samples treated with soy protein isolate at various concentrations (LISP1%, LISP2%, LISP3%) compared to the control (LC0%), highlighting the direct influence of this component on the mechanical properties of muscle tissue.

Table 5. Mean (± standard deviation) of texture parameters for the samples of injected pork tenderloins

Analyzed parameter	Sample				*p-value*
	LC0%	LISP1%	LISP2%	LISP3%	
Shear force (N/cm^2)	12.700 ± 2.209	15.593 ± 3.184	10.870 ± 2.248	11.386 ± 3.061	0.018
Work of shear (mJ)	488.516 ± 192.215	436.063 ± 62.696	318.379 ± 67.726	439.969 ± 115.028	0.017
Hardness (N)	31.953 ± 8.941	31.691 ± 6.187	21.534 ± 3.454	26.609 ± 6.991	0.079
Cohesiveness (N)	0.186 ± 0.082	0.273 ± 0.214	0.349 ± 0.235	0.241 ± 0.140	0.553
Elasticity	0.127 ± 0.022	0.156 ± 0.032	0.109 ± 0.022	0.114 ± 0.031	0.059
Gumminess (N)	6.040 ± 3.136	9.262 ± 8.542	6.962 ± 3.489	6.898 ± 4.930	0.811
Chewiness (N)	0.795 ± 0.456	1.424 ± 1.295	0.736 ± 0.348	0.880 ± 0.769	0.529
Adhesiveness	1.615 ± 0.481	0.961 ± 0.072	0.693 ± 0.044	0.660 ± 0.0318	<0.0001

Values are given as means ± Standard deviation from five repeated determinations. Means with different superscripts in a row orientation indicate significant differences ($p < 0.05$) between samples determined by the Tukey test.

The force required to shear the samples varied significantly between batches ($p = 0.018$). LISP1% batch showed the highest value (15.593 ± 3.184 N/cm²), significantly higher than LISP2% and LISP3% batches. This result indicates an increase in cutting resistance when a low percentage of protein isolate is added, an effect possibly due to protein networks formed

as a result of the interaction between the meat proteins and the soy isolate proteins. However, higher concentrations appear to decrease this resistance, possibly due to protein dilution or a more gel-like texture.

Shear-associated mechanical work values follow the same trend as the shear force ($p = 0.017$), with a maximum value in the control batch (488.516 ± 192.215 mJ), followed by a decrease in LISP1% and LISP2%. This reduction in required energy may reflect a decrease in internal structural cohesion as a result of the applied treatment.

The hardness, defined as the maximum force registered at first compression, did not show significant differences between batches ($p = 0.079$), although a clear tendency of reduction was observed in the treated batches, especially in LISP2% and LISP3%. This result is in agreement with the literature, which highlights the ability of soy protein isolate to interact with myofibrillar proteins, modifying the protein network and conferring a softer texture.

The parameters cohesiveness ($p = 0.553$) and elasticity ($p = 0.059$) did not show statistically significant differences between batches, although a slight increase in cohesiveness in the treated batches and a slight decrease in elasticity in the LISP2% batch were observed. These variations could be attributed to the reorganization of the protein networks in the presence of the isolate, but without significantly affecting the viscoelastic behavior of the product.

Gumminess was higher in the LISP1% batch, consistent with the increased values of hardness and cohesion. However, these differences were not statistically significant ($p = 0.811$). Similarly, chewiness, resulting from the product of gumminess and elasticity, followed the same pattern ($p = 0.529$). These parameters suggest that, at low concentrations, soy protein isolate contributes to a more consistent texture, while at higher concentrations, the structure becomes more brittle and chewable.

Of all parameters analyzed, adhesivity ($p < 0.0001$) was the most affected by treatment, decreasing significantly in the injected batches. The maximum value was recorded in the control batch (1.615 ± 0.481 N-s), while the values in the treated batches decreased progressively, reaching the minimum in LISP3% (0.660 ± 0.031 N-s). This decrease indicates better homogeneity and a less sticky surface, traits favored by the modified protein network and potentially of technological interest for packaging and handling.

Similar to the observations in the present study, Lin and Barbut (2025) reported that the addition of soy protein isolate influences meat textural properties and water-holding capacity, these effects being dependent on the level of substitution and initial moisture composition.

The PrefMap plot, shown in Figure 1, illustrates consumer preferences for meat samples injected with soy protein isolate (SPI) at different concentrations. By applying the external preference mapping method, the hedonic preference data are projected into the sensory space defined by the first two principal components (F1 and F2), providing a clear picture of the relationship between the sensory profile of the products and the degree of liking expressed by different consumer clusters.

The external preference mapping method was used to correlate consumers' hedonic data with the sensory profiles of the products, projected in the space formed by the first two main components (F1 and F2). The initial interpretation of the graph focuses on the positioning of the four analyzed samples (243 - control, 141 - 1% SPI, 351 - 2% SPI, 611 - 3% SPI) in distinctly colored areas of the map. These colors correspond to consumers' preference levels, where red indicates the area with the highest liking (above 80%) and blue represents the area with the lowest preference level (between 20% and 40%).

Sample 141 (1% SPI) is located in the upper-left quadrant, in the orange area (60-80% preference), close to the preference direction of Cluster 2. This suggests that the sample is moderately liked, especially by consumers with expectations oriented towards sensory characteristics such as uniform color, characteristic odor and balanced taste.

Sample 243 (control batch) is located close to the origin, but still in the orange zone. This reflects an average preference, supported in particular by consumers in Cluster 3, who appear to have a more conservative preference orientation, perhaps valuing the traditional characteristics of colour, smell and taste.

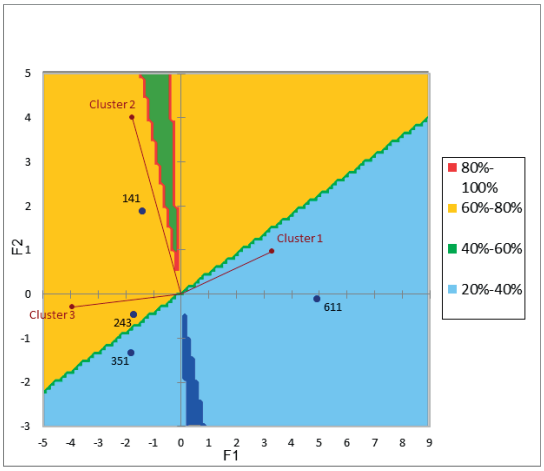


Figure 1. Positioning of the four pork chop samples injected with soy protein isolate (SPI) according to the preferences expressed by consumers in the sensory evaluation. The following samples were analyzed: 243 - control (without SPI), 141 - 1% SPI, 351 - 2% SPI and 611 - 3% SPI.

Sample 141 (1% SPI) is located in the upper-left quadrant, in the orange area (60-80% preference), close to the preference direction of Cluster 2. This suggests that the sample is moderately liked, especially by consumers with expectations oriented towards sensory characteristics such as uniform color, characteristic odor and balanced taste.

Sample 351 (2% SPI) is positioned in the blue zone, close to Cluster 3, indicating that only a small proportion of the evaluators showed appreciation. It is possible that this sample suffered in terms of taste or texture, or had an unconvincing combination of attributes.

Sample 611 (3% SPI) lies in a region marked in blue (20-40% preference), positioned on the positive F1 axis, but at a significant distance from all three preference clusters. This positioning indicates a low appreciation from all consumer segments and a possible lack of sensory alignment with their general expectations. It may be perceived as having an excessively firm texture, modified taste or exaggerated elasticity.

CONCLUSIONS

Our results suggest that injecting pork tenderloin with soy protein isolate significantly influences certain textural properties, in particular shear strength, mechanical shear work and adhesiveness. While low isolate concentrations (1%) appear to improve consistency and shear strength, higher doses lead to a decrease in these characteristics. The significant decrease in adhesiveness may be a functional advantage in the context of further product handling and processing. From a technological point of view, the adaptation of soy protein isolate concentration needs to be carefully calibrated to achieve the desired balance between consistency, palatability and manipulability.

The data obtained indicate that the addition of soy protein isolate causes significant changes in the color of the injected meat, reflected in increased brightness and decreased red intensity and color saturation. These changes may have both technological and sensory implications, as color is a critical factor in consumer acceptability. From an industrial perspective, these effects should be taken into account in recipe optimization, especially if the final product aims at a visual appearance close to that of untreated meat.

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SOIL PROPERTIES IN BRATEȘ FISH FARM: EFFECTS OF AGRICULTURAL USE ON PHYSICOCHEMICAL PARAMETERS

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Abstract

This study aimed to investigate the physicochemical properties of soil from the Brateș Fish Farm, focusing on the effects of converting fishponds into agricultural land. The research examines explicitly how aquaculture practices impact key soil properties. To achieve this, soil samples were collected using standard procedures during the summer season and stored in polythene bags. Using standard analytical methods, these samples were analyzed in the laboratory for several physicochemical parameters, including pH, electrical conductivity, total soluble salts, and the concentration of Mg^{2+} and Ca^{2+} ions. In addition, the study assessed moisture content and soil porosity to evaluate the impact of land use changes on soil quality. The results of this study provide valuable insights into the long-term effects of aquaculture on soil characteristics and offer important considerations for the future agricultural use of such converted lands.

Key words*: agricultural use, converting fishpond, physico-chemical parameters, soil.*

INTRODUCTION

Soil properties have a crucial role in keeping the ecosystem balanced as well as agricultural productivity. In a fish farm, changes in soil physicochemical properties are expected due to the involvement of food residues and fish excrement, which may affect nutrient cycling, organic carbon content, and various aspects of soil quality. Fish farming has been shown to contribute considerable amounts of organic and inorganic waste, along with nutrient loads and other residuals to the environment (Matijevic et al., 2006). These discharges may intensify nutrient enrichment (Olsen et al., 2008; Zhang et al., 2015), accelerating the process of eutrophication (Petersen et al., 2016) and soil deterioration (Mavraganis et al., 2020). Moreover, these changes primarily deteriorate water quality, adversely impact fish health and growth performance (Raman, 2018), which ultimately compromises the sustainability of fish farming. In this context, the conversion of fish ponds to agricultural land is essential to investigate soil recovery processes. Crop production, nitrogen cycles, and soil composition interact intricately throughout this transition from aquatic habitats to farmland.

This paper aimed to analyze the physicochemical parameters of soil from the Brateș Fish Farm, focusing on the effects of fishpond-to-agriculture conversion. Integrating aquaculture practices with soil properties is a necessity for sustainable land development at the Brateș Fish Farm, where fish farming and agriculture coexist. The deposition of excess nutrients due to fish waste and feed residues has led to alterations in soil pH, organic matter content, and the equilibrium level of important minerals. Therefore, the present study emphasizes important soil properties, such as soil porosity and moisture content, electrical conductivity (EC), total soluble salts (TSS), pH, alkalinity, and calcium and magnesium cation levels. By analysing these factors, the study seeks to understand the implications of previous aquaculture practices on the soil's suitability for agricultural production and to provide insights into sustainable land management strategies for similar sites.

MATERIALS AND METHODS

Study area. The Brateș Fish Farm is a facility of the Institute of Research and Development for Aquatic Ecology, Fishing, and Aquaculture, and it is dedicated to the research and implementation of specific aquaculture technologies and the production of fish with superior productive qualities. The farm is

located in the southern part of Lake Brates, near the Galati-Reni railway, in the meadow of the Prut River, in the northern part of Galați County, Romania (Figure 1) and it has a total area of 321.51 hectares, of which 292 hectares represent water surface (divided into several ponds for fish growth). Due to the declining fish productivity, it has been decided that certain ponds to be left dry for agricultural practices to improve soil properties. In this context, the present soil samples were collected after agriculture had been practiced in these fish ponds for 12 years.



Figure 1. Study area (Source: google maps)

Soil analysis. Soil samples were taken randomly from a depth of 20-25 cm using the closed-envelope method. In this sens, 500 g of soil was collected from each sampling point. All samples were collected during the summer season. The soil samples were placed in clean, labelled polyethylene bags and then transported to the laboratory for further analysis.

Table 1. Description of each sample based on the sampling stations

Sample (map point)	Land Use
S1	Grapevines
S2	Corn
S3	Vegetables
S4	Sunflower
S5	Reed

The collected samples were analyzed for the key physical and chemical soil quality parameters, including soil porosity and moisture content, electrical conductivity (EC), total soluble salts (TSS), pH, alkalinity, calcium, and magnesium cations levels.

Briefly, the soil porosity was determined by calculating the ratio of the volume of water absorbed by the material to its total volume. The result was expressed as a percentage (%) (Mureșan, 2018).

Soil moisture content was determined using the oven-drying method, as described by Su et al. (2014).

The EC of the soil was determined using a multi-parameter meter (multiEC-15). The total soluble salts content (TSS) in the soil was calculated using the following relation:

$$\text{TSS (g soluble salts per 100 g soil)} = \text{EC}\left(\frac{\text{mS}}{\text{cm}}\right) \times 0.3255,$$

where:

TSS is the total soluble salt content (g salts/ 100 g soil), and EC is the electrical conductivity (mS/cm).

The pH was determined based on the concentration of hydrogen ions (H^+) and hydroxide ions (OH^-) in the soil solution according to the procedure described by FAO (2021).

The total alkalinity of the soil was determined by the reaction of the soil solution with compounds such as bicarbonates and carbonates. It was measured by extracting an aqueous solution from the soil and titrating it with HCl (0.01 N), using methyl orange as the indicator. A color change at the endpoint indicates the alkalinity, and it is expressed as

milliequivalents H^+/100 g soil (Van Ranst et al., 1999).

Estimation of Calcium and Magnesium cations in soil samples was made according to El Mahi et al. (1987.

Statistical analysis. Statistical analysis was done using the statistical package for social science software SPSS statistical software for Windows, Version 26, United States, Chicago, SPSS Inc.

Data for the physicochemical parameters of soil samples were represented as mean values ± standard error of mean of the replicated samples (n=7). ANOVA was conducted to compare the results obtained for the physicochemical parameters of soil between the sampling points.

RESULTS AND DISCUSSIONS

Integrating agriculture practices in fish farm ponds has been recognized as a sustainable practice, especially for the recovery of soil properties. This study offers data regarding the most important physicochemical parameters and their effect on soil quality, particularly in the context of converting fishponds into agricultural land.

Determination of soil porosity and moisture content

The soil porosity and moisture content of the collected samples are presented in Figure 2. The obtained values for soil porosity indicate a variation across the different samples, with the significantly highest porosity observed in Sample 1 (13.5 ± 0.76%), where grapevines were cultivated, while the lowest porosity (9.5 ± 0.41%) was observed in S4, where sunflower was cultivated. No significant ($p>0.05$) differences were recorded in the porosity of the S2 (10.75 ±0.42 %), S3 (11.5 ± 0.74%), and S5 (10 ± 0.75%) samples, where the land was used for corn, vegetables, and reed.

Soil porosity refers to the volume of pores capable of holding water and air, which directly affects water retention, drainage, and the availability of oxygen to plant roots. The differences in soil porosity observed among the samples can be linked to different land use practices, notably the early involvement of aquaculture in the previous history of the soil. Soil provided from S1, which proved to have the highest porosity values, likely seems to benefit from natural regeneration. Generally, higher porosity increases water retention and aeration needed for plant growth, whereas low porosity indicates compaction of the soil or less space for water and air. Thus, these aspects can affect soil fertility as well as root growth. In contrast, soil from S4 likely experienced soil compaction, which suggests reduced pore spaces, hindering the soil's ability to hold water and air, potentially compromising sunflower root growth and overall soil fertility.

Regarding the moisture content of the analyzed samples, results showed significant variation with the land use ($p<0.05$). The lowest humidity content was recorded in the S4 samples (6.08 ± 0,39%), where sunflower was cultivated, while the highest humidity content was observed in the S3 samples (15.92 ± 0.47%), where vegetables were cultivated. No significant differences ($p>0.05$) were found between the moisture content of samples from grapevines (S1 – 13.63 ± 0.51%) and reeds (S6 – 13.92 ± 0.80%). The humidity content from S2 was significantly different from the other samples (7.96 ± 0.44%). In the current study, soil from S1, which exhibited higher porosity, proved to have a moderate moisture content. Therefore, it is presumed that higher porosity has allowed a fairly good water retention capacity in the soil, ensuring sufficient water availability for plant absorption. On the other hand, the lowest porosity registered for S4 aligns with the lowest moisture content, suggesting the compaction of the soil, which can negatively impact both water retention and overall moisture availability for plant roots.

Also, the differences in the moisture content of the collected samples can be explained by the organic matter content from the soil, which is influenced by the previous practice of aquaculture. Aquaculture activities typically increase the organic matter in the soil due to the addition of organic materials such as fish waste and feed residues (Ackefors et al., 1994). This increased organic matter improves the soil's ability to retain water, which could contribute to higher moisture content in certain samples (Védère et al., 2022).

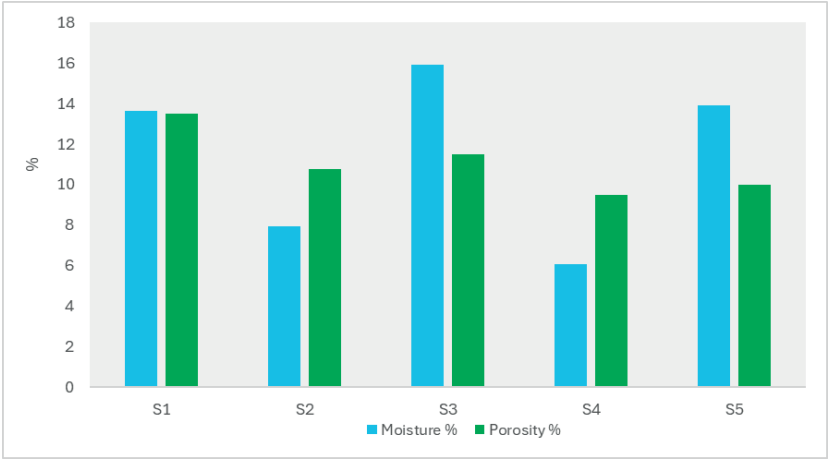


Figure 2. Soil porosity (%) and moisture content of the analyzed samples

Figure 3 presents the values of the Electrical Conductivity (EC) and the total soluble salts (TSS) for the analyzed samples. In our study, the highest value of EC was recorded in the S3 samples (0.72 ± 0.007 mS/s), followed by S5 soil (0.65 ± 0.0189 mS/s), with no significant differences ($p>0.05$). Also, no significant differences ($p>0.05$) were recorded in the values of EC for the samples S1 (0.40 ± 0.011 mS/s), S2 (0.43 ± 0.018 mS/s), and S3 (0.4 ± 0.018 mS/s).

Electrical conductivity (EC) measures the soil's capacity to conduct electric current and serves as a reliable indicator of nutrient availability. A higher EC generally suggests a greater presence of negatively charged particles, such as clay and organic matter, in the soil. Consequently, this results in increased retention of cations, such as sodium, ammonium, potassium, calcium, magnesium, hydrogen, iron, aluminum, copper, zinc, and manganese, all of which are essential for plant growth (Mureșan, 2018).

The results of our study indicate that soil from S3 and S5 samples, with higher EC, might be more effective in retaining essential nutrients, which could support plant growth. On the other hand, lower EC values, like those in S1, S2, and S4, indicate that the soil may have lower levels of dissolved nutrients, which could affect plant nutrient uptake and overall growth. According to the standard classification of EC values (Landon, 1991), soils with an EC between 0.51 and 1.25 mS/cm are considered appropriate for plant cultivation and thus, since the EC values in our study lie within this range, the soils analyzed can be classified as suitable for plant growth although those with higher EC values may retain more nutrients.

TSS refers to the total salt concentration in the soil solution and influences a range of soil parameters, like the hygroscopic coefficient, soil permeability to water, alkalinity, salinity, or soil buffering capacity (Mureșan, 2018). In our study the higher values of TSS were also recorded for the S3 (0.23 ± 0.007 g/100 g soil) and S5 (0.21 ± 0.003 g/100 g soil) samples, with significant differences ($p<0.05$) in comparison with the S1 (0.13 ± 0.011 g/100 g soil), S2 (0.14 ± 0.007 g/100 g soil) and S4 (0.14 ± 0.003 g/100 g soil). The mean values of pH, Calcium, and Magnesium cations are presented in Figure 4.

Soil pH is an important parameter that influences nutrient availability for plants, microbial activity, and soil structure and is essential for plant growth and development (El-Ramady et al., 2014; Neina, 2019; Shi et al., 2021). No significant differences ($p>0.05$) were reported in the pH values between the soil samples collected. The highest pH values were found in the S5 (7.5 ± 0.022) sample, followed by the S1 (7.26 ± 0.03), S2 (7.26 ± 0.03), S4 (7.15 ± 0.01), and S3 (7.16 ± 0.01), respectively. However, the soil pH shows alkaline conditions, suitable for plant growth. Grapevines, corn, vegetables, and sunflowers generally perform well in soils with a pH of 6.0 to 7.5. Generally, reeds tend to prefer slightly alkaline conditions but grow very well at a pH of 7.5 (Khaled and Sayed, 2023).

Regarding the Ca and Mg cations, there were significant differences ($p<0.05$) recorded between the soil samples. The calcium cation levels vary across the soil samples, with the highest value observed in S5 (14.6 ± 0.41 mg/100 g soil). S1 (7.29 ± 0.29 mg/100 g soil) and S2 (6.01 ± 0.28 mg/100 g soil) have the lowest calcium values, while S3 (12.77 ± 0.07 mg/100 g soil) and S4 (11.78 ± 0.12 mg/100 g soil) show intermediate levels. The magnesium cation concentrations also vary, with S5 (17.43 ± 0.40 mg/100 g soil) showing the highest value, similar to calcium. The higher Ca and Mg cations from S5 are further associated with the increased values of pH. When the levels of Ca and Mg increase in the soil, these cations tend to neutralize hydrogen ions (H^+), which are responsible for soil acidity. Further, the Mg cations from S2 (12.14 ± 0.35 mg/100 g soil) are significantly different from those from S3 (6.6 ± 0.04 mg/100 g soil) and S4 (6.81 ± 0.07 mg/ 100 g soil). The Mg cations registered the lowest value in the S1 (3.12 ± 0.05 mg/100 g soil). These cations are very important in the physical, chemical, and biological enhancement of the soil, its structure-related improvement, the correction of soil acidity, and the mitigation of soil salinization.

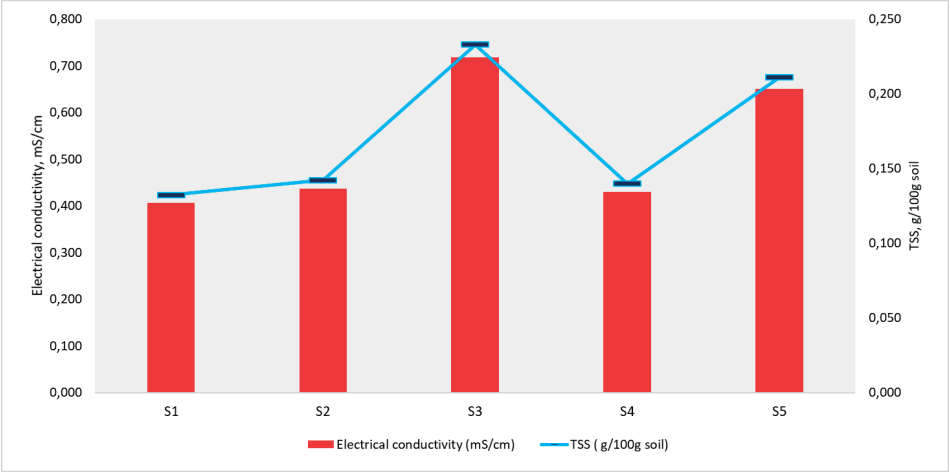


Figure 3. Electrical conductivity and total soluble salts of the analyzed samples

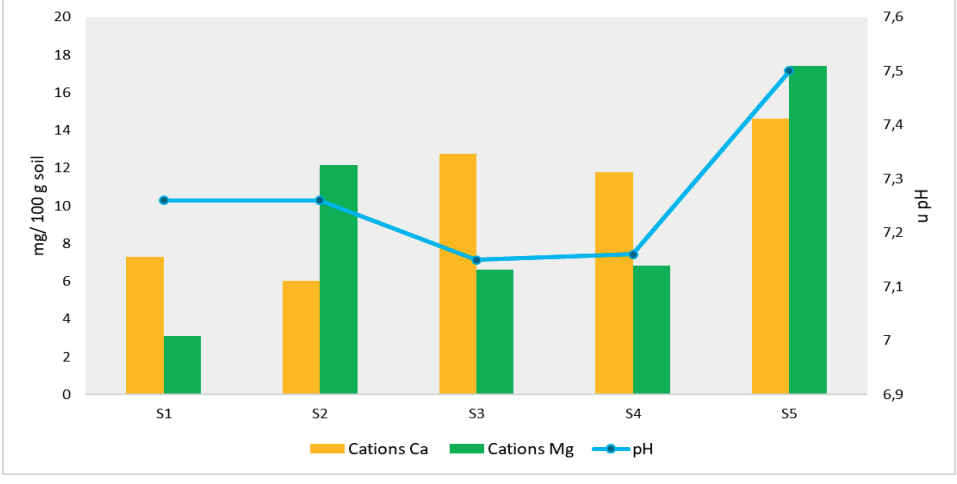


Figure 4. The pH and the Calcium and Magnesium cations of the analyzed samples

Figure 5 presents the soil alkalinity and salinity of the collected samples. Soil salinity is determined by the accumulation of soluble salts in the soil, leading to the formation of saline compounds. Alkalinity is determined by the sodium saturation of the soil's adsorptive complex, which, through reaction with water containing high levels of CO_2, generates sources of alkalinity by forming hydroxyl anions, resulting in sodic and alkaline soils (Zewd & Siban, 2021).

Alkalinity is a characteristic of the solution in saline, alkaline, and saline-alkaline soils. The alkalinity values of the soil samples ranged from 0.815 to 1.908 meq H^+/100 g soil, with the highest value recorded in sample S2 (1.91 ± 0. 003 meq H^+/100 g soil), and the lowest in sample S5 (0.81 ± 0.007 meq H^+/100 g soil). No significant differences ($p<0.05$) were recorded in the alkalinity of S3 (1.26 ± 0.007 meq H^+/100 g soil) and S4 (1.29 ± 0.007 meq H^+/100 g soil). Alkalinity reflects sodium saturation and influences soil buffering capacity. Higher alkalinity from S2 may affect plant growth by altering soil chemistry and reducing nutrient availability. In contrast, the lower alkalinity in S5 indicates better conditions for nutrient uptake.

Regarding the salinity of the soil samples, the highest salinity was recorded in the S3 (351 ± 4.18 mg/L), followed by the S5 (321 ± 3.64 mg/L). No significant differences ($p>0.05$) were recorded between S2 (210.6±3.08 mg/L), S4 (208±3.78 mg/L). The soil from S1 showed the minimum salinity (194.3±3.18 mg/L), meaning this area would accumulate very little salt due to good drainage, low evaporation, or less exposure to saline water sources.

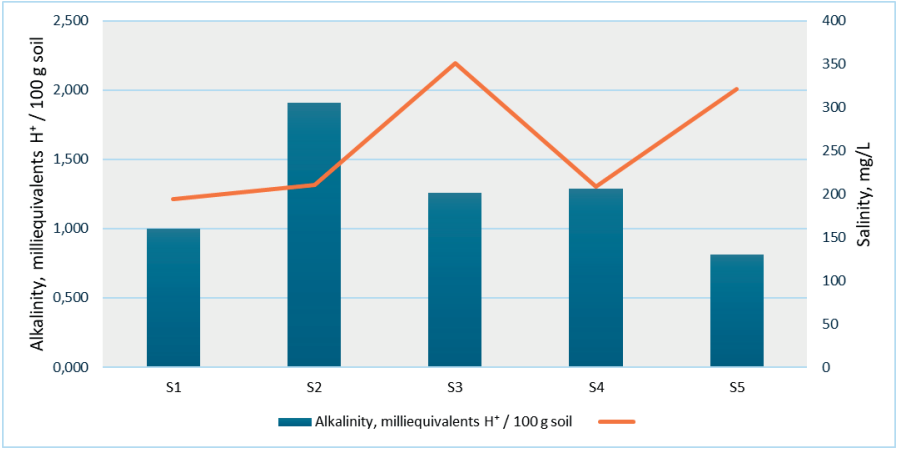


Figure 5. Alkalinity and salinity of the analyzed samples

CONCLUSIONS

This study highlights the impact of the integration of agricultural practices into fish farm ponds. Subsequently, 12 years after the fish ponds were left dry and agriculture was practiced, an improvement in the soil structure was observed. The analysis of soil physicochemical parameters demonstrates significant variations in porosity, moisture content, electrical conductivity, soluble salts, and cation concentrations, which are influenced by the previous land use practices of aquaculture activities. Certain samples show higher porosity and moisture contents, implying regeneration of the soil. It was observed that the pH of the soil samples was generally alkaline, which is good for plant growth. Calcium and magnesium cations have been found to improve soil structure and counteract acidity. Their higher concentrations in soil samples attest to suitable pH levels and improved conditions for the growth of plants. Therefore, the overall finding suggests that integrated agricultural practices in fish farm ponds can facilitate the recovery of soil quality in terms of the fertility and productivity of crops.

ACKNOWLEDGEMENTS

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EVALUATING KALE (*Brassica oleracea* var. *acephala*) GROWTH IN AN AQUAPONIC SYSTEM

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Abstract

This study explores the growth performance of kale (Brassica oleracea var. acephala) cultivated in an aquaponic system integrated with fish production. The experiment was conducted in a controlled environment using varying planting densities to assess their impact on plant development and overall system productivity. Four densities were evaluated: 14 plants/m², 21 plants/m², 28 plants/m², and 41 plants/m² respectively. Plant growth performance, including plant height, leaf number, and biomass, were evaluated across densities to determine the optimal conditions for kale growth while maintaining water quality parameters for the fish. Results showed significant differences in growth across the stocking densities, with 28 plants/m² achieving the highest productivity, marked by greater biomass and vigorous development. The findings highlight the potential of aquaponics as a sustainable cultivation method, effectively recycling fish-derived nutrients to support the growth of kale.

Key words*: crop yield, nutrients, plant growth, water quality.*

INTRODUCTION

Aquaponics is an innovative, sustainable food-growing system that combines the cultivation of plants and fish (Timmons and Ebeling, 2013; David et al., 2022; Liu et al., 2025). In these systems, fish waste is a natural fertilizer for the plants, while the plants filter and purify the water, creating a closed-loop ecosystem with minimal environmental impact. Beneficial bacteria convert fish waste into essential nutrients, which the plants absorb, thus maintaining the system's balance (Al-Hafedh et al., 2008; Filep et al., 2016). The purified water is then returned to the fish tank, completing the cycle (Rakocy et al., 2004). Aquaponics is, therefore, an extremely efficient, resource-conserving food production technique. Its water use is lower than in traditional soil agriculture, given that water is cycled through the system instead of being lost through evaporation or runoff. Additionally, the absence of soil reduces the need for massive land use and environmental degradation linked with conventional farming practices (Ibrahim et al., 2023).

Therefore, aquaponics offers a resilient and climate-smart agricultural solution in the context of increasing global water disparity and the pressing challenges of climate change. While conserving water by dramatically reducing its use and negating nutrient runoff, aquaponic systems also help protect water resources and lower the carbon footprint of food production. As such, aquaponics' efficiency and adaptability afford this strategy prominence among those required to ensure sustainable food security against environmental change, particularly when climate change threatens traditional agriculture through severe weather or resource scarcity.

However, the management of aquaponic systems is also faced with several challenges. A key aspect of aquaponics is determining the specific nutrient requirements of each plant species based on the available nutrients in the system. Monitoring water quality and nutrient

levels is crucial for maintaining a balanced and effective aquaponic system.

Kale (*Brassica oleracea* var. *acephala*), a member of the cruciferous vegetable family, has become a prominent staple in modern diets due to its exceptional nutritional profile and numerous health benefits (Šamec et al., 2019). Rich in vitamins A, C, and K and essential minerals like calcium and iron, kale is renowned for supporting immune health, promoting bone strength, and combating oxidative stress. In addition to its health advantages, kale is also a versatile crop in sustainable agricultural systems.

This study evaluates kale growth in an aquaponic system, exploring the plant's nutrient needs and performance within this sustainable farming approach.

MATERIALS AND METHODS

Experimental design. The experiment was conducted at the Romanian Centre for Modelling Recirculating Aquaculture Systems (MoRAS- www.moras.ugal.ro) of the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galați, Romania. The description and functioning of the aquaponic system were previously presented in our article (Crețu et al., 2022).

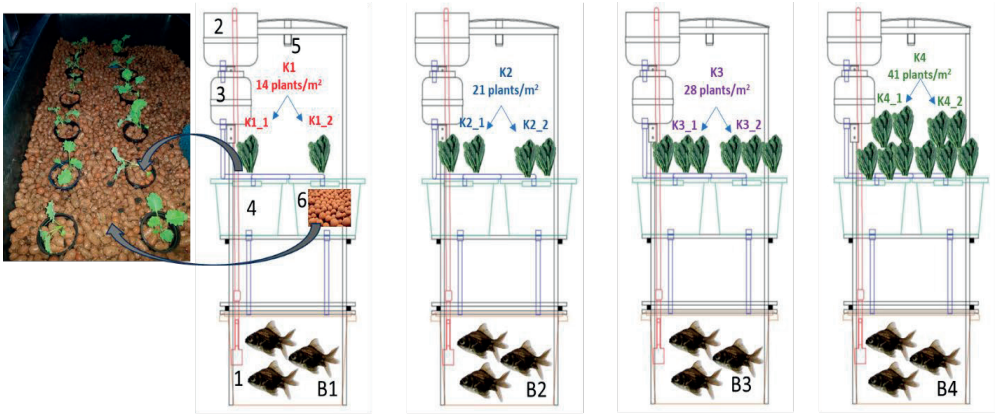


Figure 1. The scheme of the aquaponic system:
B1-B4 - fish rearing unit; 1- recirculation pump; 2- mechanical filter; 3- biological filter;
4- aquaponic units; 5- led lamp; 6- Lightweight Expanded Clay Aggregate (LECA) substrate

For this experiment, we used 33 fish per tank (B1-B4) (Figure 1) (*Carassius auratus* var. Black Moor), with an average size ± SEM of 12.67 ± 0.07 g. Fish were fed manually with extruded pellets (34% protein, 15% lipids, 1.4% crude fibre, 6.80% ash). The fish were fed daily at a rate of 5% of their total biomass, with the total feed amount distributed across three meals at 8:00 a.m., 12:00 p.m., and 6:00 p.m.

The kale seedlings (Figure 2) were obtained in our laboratory. Seedlings were irrigated with a nutritive solution (0.27% Fe, 0.07% Mn, 0.05% Zn, 0.04% B, 0.009% Cu, and 0.006% Mo) to support plant growth (2 mL solution/L water). After true leaves were developed (4-5 leaves), plants were transferred to the aquaponic system (after 25 days). The following experimental variants were created, in duplicate: K1 - 14 plants/m^2, K2 - 21 plants/m^2, K3 - 28 plants/m^2 and K3 - 41 plants/m^2.



Figure 2. Kale seedlings before transfer to the aquaponic system (Original photo)

All the plant units were filled with Lightweight Expanded Clay Aggregate (LECA). LECA is a porous, lightweight material used in aquaponic

systems as a growing medium. It is chemically inert and durable and doesn't affect the system's pH or nutrient levels. It provides excellent aeration and drainage for plant roots while retaining sufficient water.

Water quality analysis, including temperature (°C), dissolved oxygen (mg L^{-1}), and pH, were monitored daily using a Pro1020 (YSI Incorporated, USA) and pH-meter (WTW InoLab pH 7110, Xylem Analytics, Germany). Nitrogen compounds (nitrates, nitrites, and ammonium) were measured twice weekly with Merck kits and a Merck Spectroquant Nova 400 spectrophotometer (Merck Chemicals GmbH, Germany). Water samples were taken at different points: after mechanical and biological filtration (M+B), from the plants growing units (P), and the fish basins (F). Luminous intensity (lx) was measured weekly with a TESTO 545 light meter (Testo Co., USA).

Plants and fish growth parameters. The physical growth of kale was determined by measuring weight gain of plants (g) (WG = final biomass of plants-initial biomass of plants), leaf area surface (cm^2/plant), final roots weight (g), and the number of leaves. At the end of the experimental trial, fish growth parameters such as feed conversion ratio (FCR), specific growth rate (SGR), feed intake (FI), body weight gain (BWG), and survival (S%) were calculated as follows:

- FCR = FI/BWG;
- SGR = (ln (Final body weight) − ln (Initial body weight))/Number of days;
- BWG = Final body weight − Initial body weight;
- FI = amount of feed provided – amount of remaining feed;
- S = (Number of fish at the end of the study/Number of fish at the beginning of the experiment) × 100.

Plant analysis. Plants were dried in a ventilated oven (Jeiotech, Jeio Tech Co., Inc., Korea) at 60°C until a constant weight was achieved, allowing for the determination of dry weight in grams. This value was then used to calculate the dry matter (DM) percentage. Nitrite and nitrate levels in plants were determined by the Griess method (STAS 9065:2002).

The chlorophyll content was assessed by extracting chlorophylls from leaf tissues using ethanol, following the procedure outlined by Castle et al. (2011). Absorbance was measured at specific wavelengths corresponding to chlorophyll a, b, and total chlorophyll, specifically at 649 nm and 665 nm.

The phosphorus concentration was measured colorimetrically using the SANseries Automated Wet Chemistry Analyzers (SANseries Skalar Analytical B.V., The Netherlands). The phosphate determination method relies on the reaction between ammonium heptamolybdate and potassium antimony (III) oxide tartrate in an acidic solution, with phosphate ions forming an antimony-phosphomolybdate complex. This complex is subsequently reduced by L(+) ascorbic acid, resulting in a deep blue-colored complex, which is quantified spectrophotometrically at 660 nm.

Statistical analysys. The data collected were processed using IBM-SPSS statistical software and presented as Mean ± SEM. Prior to conducting analysis of variance (ANOVA), Levene's test was applied to assess homogeneity of variances. One-way and two-way ANOVA were then used to evaluate significant differences in the parameters measured. Differences between means were determined using the Duncan multiple range test with a significance level set at $\alpha = 0.05$.

RESULTS AND DISCUSSIONS

Water quality parameters. In the experiment, maintaining water quality within optimal ranges was crucial for the health and growth of kale plants and fish, ensuring the success of the aquaponic system. The water temperature was kept within the ideal range for kale growth (Table 1), aligning with the findings of Chowdhury et al. (2021) that suggest a preferred temperature range of 20°C to 23°C, with a broader tolerance of 18°C to 28°C in greenhouse environments (Catigday et al., 2023). Additionally, dissolved oxygen levels remained consistent, with no significant differences between fish tanks ($p > 0.05$), ensuring both fish and beneficial bacteria had adequate oxygen for respiration and nitrification. The water pH ranged from 7.24 to

7.55, which was optimal for fish and plants, promoting a stable environment for the essential nitrification process, supporting nutrient cycling in the system.

The nitrogen compounds ($N\text{-}NO_2^-$, $N\text{-}NH_4^+$, and $N\text{-}NO_3^-$) did not show significant differences between fish tanks ($p > 0.05$), but their levels decreased significantly after water evacuation from the plant units ($p < 0.05$). This decrease is attributed to the plant's ability to absorb and retain these nitrogen compounds, highlighting their critical role in the nitrogen cycle. By taking up ammonium and nitrate, plants help regulate nutrient levels, reducing the concentration of potentially harmful compounds in the water.

Table 1. The physicochemical parameters of water during the experimental trial

Parameter	Fish tanks				Alimentation of aquaponic units				Evacuation of aquaponic units			
	B1	B2	B3	B4	K1	K2	K3	K4	K1	K2	K3	K4
Temperature (°C)	20.8± 0.16	20.7± 0.21	21.06± 0.23	20.9± 0.31	21.02± 0.4	21.12± 0.24	20.9± 0.25	20.6± 0.18	21.0± 0.23	21.2± 0.24	21.1± 0.30	20.9± 0.18
Oxygen (mg/L)	7.16± 0.23	7.24± 0.21	7.32± 0.19	7.16± 0.13	-	-	-	-	-	-	-	-
pH (pH units)	7.55± 0.51	7.42± 0.49	7.34± 0.40	7.37± 0.40	7.43± 0.44	7.32± 0.28	7.31± 0.43	7.28± 0.54	7.25± 0.50	7.32± 0.47	7.24± 0.35	7.36± 0.37
$N\text{-}NO_2^-$ (mg/L)	0.04± 0.003	0.05± 0.002	0.04± 0.003	0.06± 0.002	0.05± 0.002	0.04± 0.002	0.03± 0.001	0.04± 0.002	0.02± 0.003	0.01± 0.004	0.02± 0.002	0.02± 0.001
$N\text{-}NH_4^+$ (mg/L)	1.33± 0.51	1.09± 0.46	1.66± 0.29	1.46± 0.29	1.27± 0.5	1.02± 0.48	1.08± 0.37	1.05± 0.29	0.71± 0.41	0.86± 0.52	0.76± 0.36	0.96± 0.29
$N\text{-}NO_3^-$ (mg/L)	39.74± 1.53	37.74± 1.77	37.88± 0.69	36.73± 0.38	37.05± 1.31	35.57± 1.01	32.12± 0.77	35.3± 2.17	28.46± 1.47	29.55± 1.35	28.06± 0.85	26.12± 0.73
$P\text{-}PO_4$ (mg/L)	5.77± 0.62	5.87± 0.87	5.76± 0.72	5.99± 0.62	5.71± 0.79	5.55± 0.40	4.78± 0.40	5.62± 0.34	4.12± 0.42	3.82± 0.40	4.03± 0.72	4.12± 0.25
Conductivity (µS/cm)	1921.2± 173.63	2004.4± 172.67	1956.8± 114.35	1937.75± 151.19	1942.5± 173.63	2004.75± 148.60	1943.01± 115.14	1902.12± 110.01	1829.60± 137.60	1976.25± 114.23	1896.04± 112.03	1896.12± 112.01

Note: Values are presented as Mean ± SEM (standard error of mean); B1- B4 – fish rearing units; K1-K4 – aquaponic units.

Electrical conductivity (EC), which ranged between 1829.60 and 2004.75 µS/cm, was within the optimal range for plants and fish. Lower EC values were recorded after water evacuation from the plant units, reflecting the plants' uptake of dissolved nutrients.

Maintaining proper EC levels is essential for plant nutrient uptake and preventing toxic concentrations of salts or minerals.

The balanced fish-to-plant stocking density and careful monitoring of water quality parameters contributed to the aquaponic system's efficient nutrient cycling and optimal performance.

This balance is essential for preventing nutrient deficiencies and excesses, ensuring the sustainability and productivity of the system. Careful monitoring and adjusting water quality and plant density are key to ensuring a successful and productive aquaponic system.

Plant Growth in an Aquaponic System. Table 2 present the plant growth parameters of kale in the aquaponic system at different stocking densities of plants.

The initial weight of kale registered no significant ($p>0.05$) differences between the four experimental variants (Figure 3).

After 50 days, kale's final weight was significantly higher at the density of 28 plants/m² (K3), while no differences were recorded between the densities of 14 plants/m², 21 plants/m², and 41 plants/m², respectively (Figure 4).

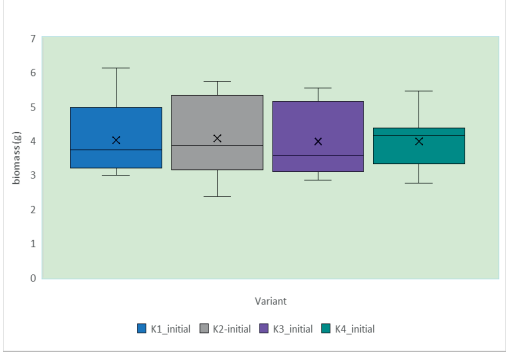


Figure 3. The distribution of the initial weight of kale

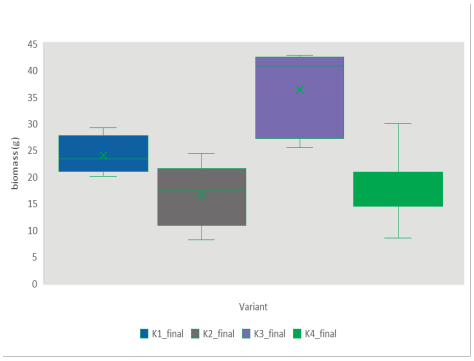


Figure 4. The distribution of the final weight of kale

The foliar surface area is a key indicator of a plant's ability to capture light and perform photosynthesis (Patil et al., 2018).

A larger leaf area allows for greater light interception, enhancing the plant's photosynthetic efficiency.

As a result, the foliar surface is closely linked to plant growth and biomass accumulation, providing valuable insight into the plant's developmental capacity and overall productivity.

At the initial stage of the experiment, the leaf area of kale plants was similar ($p>0.05$) across all four experimental variants (Figure 5).

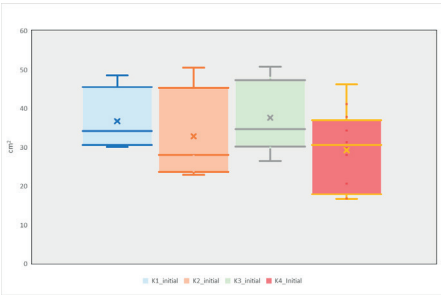


Figure 5. Leaf area of kale at the initial moment

However, at the end of the experiment, the leaf area values in the K3 variant (28 plants/m²) were significantly higher compared to the other planting densities (Figure 6).

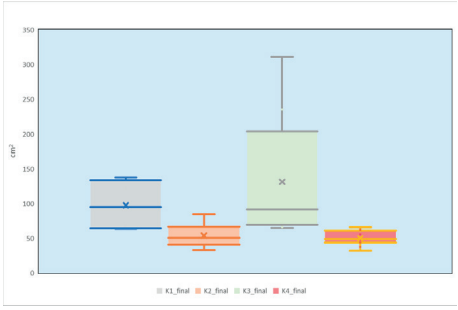


Figure 6. Leaf area of kale at the final moment

Table 2. Plant growth data from the aquaponic system

Parameter	K1 (14 plants/m²)	K2 (21 plants/m²)	K3 (28 plants/m²)	K4 (41 plants/m²)
Individual weight at initial moment (g)	4.01±0.61*	4.06±0.49*	3.98±0.35*	3,97±0.41*
Individual weight at final moment (g)	24.02±1.76*	16.64±1.56*	36.35±1.24**	17.67±2.45*
Initial biomass (g)	20.07±1.52	24.41±1.43	21.85±1.14	27.35±1.16
Final biomass (g)	96.08±3.52	99.84±2.43	290.86±2.23	212.06±3.35
Plant's weight gain (g)	76.00±2.54	75.44±2.40	269.01±2.35	184.72±2.27
Individual root weight at the final moment (g)	3.13±1.16*	7.92±1.14**	16.95±1.15***	3.5±1.09*
Number of leaves	15.63±2.16*	16.41±3.05*	23.93±1.01**	16.79±1.23*

Overall, the final plant biomass was higher in the K3 variant, followed by the K4 variant, where we have the highest plant stocking density. Regarding the number of leaves, a significantly higher number was reported in the K3 variant. The number of leaves on a plant is an important indicator of its growth, health, and productivity. A higher leaf count typically reflects strong vegetative growth, enhancing the plant's photosynthetic capacity and overall energy production.

In this study, stocking density significantly affected kale growth in an aquaponic system. The 28 plants/m² density (K3) resulted in the highest final plant weight, leaf area, and number of leaves, suggesting it is the optimal density for kale growth. At this density, plants had enough space to develop larger leaves, enhancing their photosynthetic efficiency and overall biomass accumulation. In contrast, higher densities, such as 41 plants/m² (K4), did not show significant improvements in biomass,

likely due to increased competition for light and nutrients.

These findings highlight the importance of balancing stocking density to optimize plant growth and productivity in aquaponic systems. In the current scientific literature, there is a scarcity of studies that directly investigate the effect of different plant densities on kale growth in aquaponic systems, which limits the possibility of direct comparisons and highlights the need for further research in this area.

However, our findings align with previous research conducted in hydroponic systems. For example, Noboa et al. (2022), studying curly kale in a hydroponic NFT system, observed that reducing plant spacing increased overall yield per area; however, fewer plants per cell led to larger and more uniform leaves, highlighting a trade-off between maximizing yield and maintaining product quality. Although both studies emphasize the significance of density optimization, the aquaponic system in the present study revealed stronger limitations at higher densities, likely due to the compounded effects of light and nutrient competition inherent to integrated plant-fish systems. Similarly, in another aquaponic study (Afolabi, 2020) where kale was grown alongside Nile tilapia at a fixed density of 25 plants/m², researchers highlighted that system design and configuration (integrated aqua-vegeculture vs. deep-water culture) also play critical roles in determining plant and fish productivity, as well as water quality.

In Table 3 are presented the mean values of the dry matter content (%), Chlorophyll A and B, nitrite (mg/kg), nitrate (mg/kg), and phosphorus (mg/kg) content from kale plants at the end of the aquaponic trial. Dry matter content was slightly higher at K2, indicating slightly better biomass accumulation. Chlorophyll A levels were highest in K3, suggesting enhanced photosynthesis, while Chlorophyll B increased from K1 to K3, with K3 showing the highest concentration. Regarding nitrite, K3 had the lowest concentration, reflecting efficient conversion or uptake, while K1 and K2 had higher nitrite levels. Nitrate levels were highest in K2, suggesting greater nitrification but possibly reduced plant uptake. K3 had the lowest nitrate concentration.

Finally, phosphorus concentrations increased from K1 to K4, with K3 showing the highest values, suggesting better nutrient uptake and root development. Overall, K3 seems to support efficient nitrogen conversion and photosynthesis.

Table 3. Plant analysis at the end of the trial

Parameter	K1	K2	K3	K4
Dry matter (%)	8.55±0.99	8.88±0.78	7.78±0.54	8.29±0.87
Chlorophyll A (μg×g^{-1})	0.55±0.16	0.59±0.12	0.66±0.09	0.58±0.63
Chlorophyll B (μg×g^{-1})	0.40±0.09	0.74±0.07	0.81±0.08	0.68±0.05
Nitrite (mg/kg)	0.32±0.02	0.26±0.04	0.025±0.02	0.26±0.04
Nitrate (mg/kg)	3.12±0.17	4.23±0.18	2.63±4.36	3.63±2.19
Phosphorous (mg/kg)	2.06±0.56	2.36±0.42	2.49±1.02	2.21±1.16

Fish growth performance. At the end of the aquaponic experiment, the fish survival rates were very high, ranging from 96.97% to 100%, indicating a good water quality and system management (Table 4).

Table 4. Fish growth performance at the end of the experiment

Parameter	B1	B2	B3	B4
Initial number of fish	33	33	33	33
Initial biomass (g)	416	418	417	422
Initial fish weigh (g)	12.61	12.67	12.64	12.79
Final biomass (g)	648	687	689	643
Final fish weight (g)	20.25	20.82	20.88	19.48
Survival (%)	96.97	100	100	100

The final fish biomass increased in all groups, with the highest values recorded in B2 (687 g) and B3 (689 g).

These two variants also showed the highest individual final weights, reaching 20.82 g and 20.88 g respectively, suggesting that the stocking density of plants in these variants may have created favourable conditions for fish growth, likely through better nutrient cycling and system balance.

Despite a slightly lower final biomass in B4 (643 g), survival remained at 100%, indicating no lethal stress, though growth was slightly reduced, possibly due to competition or less

optimal nutrient conditions caused by the higher plant density.

CONCLUSIONS

This study underscores the importance of carefully balancing plant stocking densities in aquaponic systems to optimize both plant growth and fish productivity.

The variant with 28 kale plants/m² emerged as the optimal density for kale growth, as it facilitated larger leaf area and better biomass accumulation.

Regarding the fish growth, the best growth performance was reported in the B3 basin (corresponding to the aquaponic units with 28 plant/m^2) and B2 (corresponding to the aquaponic units with 21 plant/m^2), likely due to the nutrient balance fostered by these plant densities.

The study emphasizes that higher plant densities beyond a certain threshold could lead to competition for light and nutrients, potentially limiting growth and productivity in both plants and fish.

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SEX STRUCTURE AND FECUNDITY OF PONTIC SHAD (*Alosa immaculata*) IN THE ROMANIAN SECTOR OF THE DANUBE RIVER

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Abstract

*The Pontic shad (Alosa immaculata), a migratory fish species of significant ecological and economic importance, is a key component of the ichthyofauna in the Danube River and Black Sea. This study investigates the sex structure and fecundity of Alosa immaculata during its breeding migration, focusing on specimens from the Romanian sector of the Danube River. Sampling was conducted from March to May every year from 2020 to 2024, coinciding with the peak reproductive season. Biological parameters like total length, body weight, and gonad weight will be recorded, while age determination will rely on scale analysis. Absolute individual fecundity (F) will be determined using the weighing method. Relative fecundity (RF) will be calculated by: RF = F*GW/ws, where GW = the gonadal weight of fish and ws = the weight of the sample. The study seeks to fill critical gaps in understanding the reproductive biology of A. immaculata, essential for developing conservation strategies and sustainable fisheries management. The findings will provide insights into population dynamics, aiding in the preservation of this species amid increasing anthropogenic pressures and environmental changes.*

Key words*: conservation, fisheries management, prolificity, reproductive biology, sex ratio.*

INTRODUCTION

The Pontic shad (*Alosa immaculata* Bennet, 1835), a migratory fish species of the Clupeidae family, plays a crucial ecological role as a key component of the ichthyofauna and holds significant economic importance for the Danube–Black Sea region (Țiganov et al., 2016; Leonov et al., 2022; 2023). Its anadromous life cycle, characterized by migrations from marine to freshwater environments for reproduction, is essential for maintaining population stability and ecological balance. This migration ensures the species' survival by providing optimal conditions for larval development and contributes to biodiversity by transferring nutrients between marine and freshwater ecosystems. Additionally, it facilitates genetic diversity, preventing inbreeding and strengthening the population's resilience to stress factors (Tamario et al., 2019).

Historically, *A. immaculata* undertook extensive upstream migrations, reaching spawning areas up to 1,000 km from the river's mouth. However, anthropogenic pressures such as overfishing, water pollution (Năvodaru & Waldman, 2003; Višnjić-Jeftić et al., 2010), reduced water levels (Năvodaru & Waldman, 2003; Smederevac-Lalić et al., 2018), and the construction of the Iron Gates I and II dams have significantly disrupted these migratory routes. As a result, the species is now largely restricted to the lower Danube River, limiting access to its traditional spawning grounds (Năvodaru, 1996; Mocanu et al., 2020). These disruptions have negatively impacted its reproductive success, a critical factor for

population sustainability (Višnjić-Jeftić et al., 2013).

According to official data from the Romanian National Agency for Fisheries and Aquaculture, Pontic shad catches have declined from 634.5 tons in 2019 to 271.25 tons in 2023 (*NAFA, 2022*). A similar decline in catches was reported by Raikova-Petrova et al. (2013) and Višnjić-Jeftić et al. (2013) in the Bulgarian sector of the Danube River. The same goes for the Pontic shad captures from the Dniester River, in the Moldavian Republic (Gologan, 2021), suggesting a possible trend of *A. immaculata* population decline, at large scale. These findings highlight the urgent need for an integrated conservation approach among riparian countries to prevent further population decline and ensure the long-term viability of this ecologically and economically important species.

Fecundity is a fundamental concept in reproductive biology, referring to an organism's potential reproductive capacity, typically measured by the number of eggs produced per reproductive cycle (Lambert, 2008). It is influenced by both intrinsic and extrinsic factors, including species - specific reproductive strategies, body size, age, nutritional status, and environmental conditions such as water temperature, food availability, and habitat quality (Murua et al., 2003; Raikova-Petrova et al., 2013).

The lifespan of *A. immaculata* is estimated at 7-8 years, with sexual maturation occurring at 3-4 years of age. Only a small fraction of individuals spawn in two consecutive seasons (Năvodaru & Năstase, 2014; Țiganov et al., 2016). In most studies, fecundity is expressed as potential annual fecundity.

Despite the ecological and economic significance of *A. immaculata* in the Romanian sector of the Danube River, there is a lack of detailed research on its reproductive biology, particularly regarding sex structure and fecundity. These knowledge gaps hinder the development of effective conservation strategies and sustainable management practices, especially in the face of increasing anthropogenic and environmental pressures.

This study investigates the sex structure and fecundity of *A. immaculata* during its breeding migration in the Romanian sector of the Danube River. Spawning occurs between late February and early March when water temperatures reach approximately 5-6°C (Năvodaru, 1997), coinciding with the seasonal rise in Danube water levels (Ciolac, 1998; 2004).

MATERIALS AND METHODS

The Pontic shad (*Alosa immaculata*) specimens were collected during scientific fishing surveys conducted by the I.C.D.E.A.P.A. Galați researchers in the Chiscani area, Braila County (Figure 1), located in Sector 2 of the Danube River, between Braila (km 169) and Gropeni (km 197). This area represents a critical habitat for the species, serving as an essential spawning ground, as well as a key migratory corridor.



Figure 1. Chiscani Area – Study zone (Source: https://browser.dataspace.copernicus.eu/)

Data Collection. Sampling was carried out annually from March to May, spanning the years 2020 to 2024, coinciding with the peak reproductive season. A total of 1,554 individuals were captured using gillnets with a mesh size of 30-35 mm. The specimens were selected through a random sampling method from commercial catches, ensuring the inclusion of individuals from all length classes. Total length (TL, cm) was measured with an ichthyometer, with an accuracy of 0.1 cm. The total weight (TW, g) and gutted weight (w, g) were determined using an electronic weighing scale with a precision of 0.01 g. Gonads were weighed with an accuracy of 0.0001 g. Length classes were established at 2 cm intervals.

Age determination was made with the use of scales collected from the anteromedial part of the body, above the lateral line. Therefore, using a stereomicroscope with a magnification of 1 x 10, the rings obtained on the scales were counted indicating the annual growth (Yilmaz and Polat, 2002).

Statistical Analysis. Statistical analyses were performed using XLStat Basic 2024.4.0 and R 4.2.2 software, following Pearson (1900) and Berk & Jones (1979).

Sex Ratio Analysis. The sex ratio was calculated annually, expressing the number of females relative to the number of males (e.g., 1:1, 2:1). The Chi-square test was employed to assess deviations from the expected 1:1 ratio (Raikova-Petrova et al., 2013; Rashid, 2018). The test compares observed and expected frequencies using the formula:

$$\chi^2=\sum\frac{(Oi-Ei)^2}{Ei},$$

where *Oi* represents the observed frequency and is *Ei* the expected frequency (calculated as 50% of the total population). The calculated values were compared with critical values at a confidence level of 0.05 and df = 1. If the calculated χ^2 value exceeded the critical value, the null hypothesis was rejected, indicating a significantly different sex distribution in the population. The obtained results were then interpreted to assess the balance or imbalance of the sex distribution within the studied population.

Absolute and relative fecundity. Individual fecundity, the number of eggs for one gram of sample was determined by the gravimetric method.

The absolute fecundity (F) was calculated using the formula: F = No. of eggs* GW/ws, where GW is the gonadal weight and ws is the sample weight (Alam & Pathak, 2010; Das et al., 2016; Roy et al., 2014), in our case 1 g/sample, while relative fecundity was determined by RF = F/TW (kg).

The normality of relative fecundity data was tested using the Shapiro-Wilk test. If $p<0.05$, the null hypothesis (H_0) of normal distribution was rejected, indicating non-normal data. Due to non-normal distribution, for both absolute and relative fecundity, which displayed a flat distribution with extreme values and negative skewness, suggesting that most values are concentrated in the upper part of the distribution, but there are also a few significantly smaller values that influence the mean, the Kruskal-Wallis test was applied to determine significant differences between years (Maruska & Fernald, 2010).

The Kruskal-Wallis test is a non-parametric test used to compare the medians of two or more independent groups. The test was chosen to assess whether there are significant differences between the distribution of fecundity values across different annual groups (Siegel & Castellan, 1988; Corder & Foreman, 2009). Post-hoc analysis was conducted using Dunn's test to precisely identify which groups are significantly different and Bonferroni correction was applied to minimize Type I errors (Dunn, 1964; Costa et al., 2015).

The gonadosomatic index (GSI) was calculated to assess reproductive condition using the formula: GSI (%) = GW/TW*100 (Hasan et al., 2020). Normality was verified via the Shapiro-Wilk test ($p>0.05$ for all years) and variance homogeneity was confirmed using Bartlett's test ($p>0.05$, $\chi^2 = 7.52 < \chi^2_{critical} = 9.49$). Further analysis included ANOVA Single Factor (df1 = 4, df2 = 81, $\alpha = 0.05$), where a significant result ($\hat{F} = 3.94 > F_{critical} = 2.48$) indicated heterogeneity among years. Tukey's test was then used to identify specific differences ($\alpha = 0.01$).

Additionally, the relationship between absolute fecundity and morphometric parameters were established to determine how reproductive output varies with body size, providing insights into the factors influencing fecundity and the overall reproductive potential of *Alosa immaculata*. These relationships help assess the impact of biological and environmental factors on egg production, contributing to a better understanding of population dynamics and the development of effective conservation and management strategies.

RESULTS AND DISCUSSIONS

Out of a total of 1554 specimens, 838 were females and 716 were males, suggesting a significantly imbalanced sex distribution within the studied population, with a higher number of females compared to males (53.93% females and 46.07% males). The most numerous females belong to the length class of 29-30.9 cm and are 4 years old – 168 specimens.

The same length and age class also contains the highest number of males – 170 specimens (Table 1).

Relatively small length classes were found by Rozdina et al. (2025) in the Black Sea, up to 20.9 cm. The age distribution revealed that most individuals are found in the 3-5 age groups, unlike previous years when the migration age for reproduction ranged from 2-7 years (Năvodaru & Waldman, 2003). Similar results were observed by Năstase et al. (2019) for the year 2016, with most of the spawner specimens in the Danube Delta falling into the 2-5 age classes, and by Crepis et al. (2018) in the Dniester River, where most specimens were between 4-5 years old.

In contrast, Raikova-Petrova et al. (2013) observed a predominantly younger population in 2010-2011 in the Bulgarian section of the river, with individuals in the 2-3 age classes. Sporadically, 8-year-old specimens were found (3 females and 1 male).

Table 1. Total distribution of Pontic shad individuals by sex, length classes, and age

Size classes (cm)	Age (t, years)														TOTAL
	2		3		4		5		6		7		8		
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
15-16.9	2	0	0	2	0	0	0	0	0	0	0	0	0	0	4
17-18.9	0	2	7	5	0	0	0	0	0	0	0	0	0	0	14
19-20.9	1	2	0	2	2	0	0	0	0	0	0	0	0	0	7
21-22.9	0	0	0	7	0	0	0	0	0	0	0	0	0	0	7
23-24.9	1	2	1	1	1	1	0	0	0	0	0	0	0	0	7
25-26.9	0	4	5	8	1	17	0	7	1	0	0	0	0	0	43
27-28.9	2	0	5	72	37	98	19	63	0	4	0	2	0	0	302
29-30.9	4	7	36	54	168	170	73	74	21	11	2	0	0	1	621
31-32.9	0	3	22	9	133	41	107	28	20	6	0	0	3	0	372
33-34.9	0	0	7	3	51	8	65	2	13	0	2	0	0	0	151
35-36.9	0	0	0	0	14	0	4	0	5	0	0	0	0	0	23
37-38.9	0	0	0	0	0	0	1	0	1	0	0	0	0	0	2
39-40.9	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
TOTAL	10	20	83	163	408	335	269	174	61	21	4	2	3	1	1554

These data suggest that the population is concentrated in the younger age groups, indicating an active and rapid development phase. In the 2-3 age classes, males dominate, while in the 4-8 age classes, females are the majority.

Sex Ratio Analysis

Through the application of the χ^2 test, we obtained a χ^2 value of 9.58, which is significantly higher than the critical value of 3.841 (for a 0.05 significance level and 1 degree of freedom). Therefore, the null hypothesis is rejected, indicating that the sex distribution is not balanced, suggesting a predominance of one gender in the analyzed sample, in this case, females.

These observations suggest an active and young population, with a significant concentration in medium and long-length intervals, accompanied by a predominance of females. The ratio of females to males in the studied population shows a slight predominance of females, with a ratio of approximately 1.17 females for each male (53.93% females and 46.07% males).

A relatively low ratio (M/F = 0.51) compared to the results of this study was observed in 2016 at the Danube Delta mouth into the Black Sea by Năstase et al. (2019), indicating the presence of 2 females for each male. Similar results were also reported by Rozdina et al. (2025) in the Bulgarian section of the Danube, with a M/F ratio of 1:2.6. High values were also obtained by Crepis et al. (2018), with an M/F ratio of 1:6 for the Dniester River. In contrast, Raikova-Petrova et al. (2013) observed a clear dominance of males in 2010-2011, with a ratio of 5.61:1, highlighting a significant shift in the structure of the population in the last decade. Furthermore, a higher female ratio may contribute to an increase in the overall fecundity of the population.

The sex structure remains consistent across different length and age classes, without significant variations between age groups, suggesting that the sex imbalance is not caused

by age or length-specific selection factors. The sex ratio depends on the catch period, as the migration seems dominated by males at the beginning of migration, coming to balance during the peak and dominated by females at the end of the process (Lambert et al., 2001).
Analyzing the M/F ratio over the years (Figure 2), it is observed that in most cases, females outnumber males, with ratios ranging from near equality (2023) to a greater imbalance in favor of females (2022 and 2024).

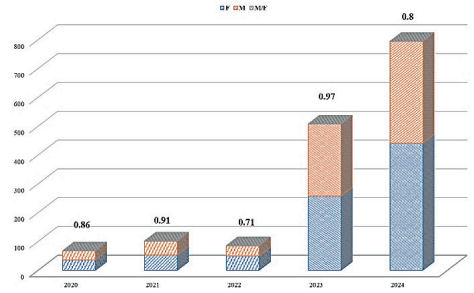


Figure 2. Sex Ratio Trend for Pontic shad between 2020 and 2024 (Source: original)

This suggests that various factors could be influencing the observed sex ratio. Another possible hypothesis could be related to external factors influencing the sex distribution. For example, environmental conditions in the studied regions (such as water temperature or resource availability) might favor a certain sex ratio depending on developmental or survival factors. These effects could vary between the studied years or based on the locations from which the individuals originate. Moreover, the influence of external pressures such as fishing practices or ecological disturbances could alter the balance between the sexes. These factors might vary over time, contributing to the fluctuations observed in the M/F ratio.

Absolute and relative fecundity

For the fecundity analysis conducted between 2020 and 2024, 86 samples aged between 2 and 4 years, with varying weights and lengths, were included. Individual fecundity varied significantly, with a minimum of 4,985 eggs/g recorded in 2022 for a 3-year-old specimen weighing 196 g and measuring 27.9 cm in total length (TL), while the maximum value of 19,213 eggs/g was observed in 2021 for a 5-year-old individual weighing 305 g and measuring 30.4 cm in TL. The average individual fecundity during the study period was 9,260.79 eggs/g ± 331.74 eggs/g, in contrast to the average obtained by Năvodaru & Waldman (2003), which was 3,122 eggs/g.
As shown in Table 2, the average values for the absolute fecundity situated ranged from 103880.9 eggs/g ± 25068.7 eggs/g in 2024 and 160441.8 eggs/g ± 56592.5 eggs/g in 2020, while the relative fecundity was situated between 376190 ± 104095.5 in 2023 and 593197.5 ± 71777 in 2022.

Table 2. Statistical parameters for absolute and relative fecundity between 2020 and 2024

	Absolute fecundity				
	2020	**2021**	**2022**	**2023**	**2024**
Mean	160441.8±56592.5	142904.3±40747.1	100902.6±15158.3	117288.6±49549	103880.9±25068.7
Minimum	56584.80	84869.95	75731.32	29539.13	70292.04
Maximum	283131.80	245004.75	128729.30	183136.40	161220.76
	Relative fecundity				
	2020	**2021**	**2022**	**2023**	**2024**
Mean	521176.2±62609.2	593197.5±71777	407121.9±29986	376190±104095.5	402331.1±28218.4
Minimum	420540.65	451435.90	369081.21	167812.89	351885.17
Maximum	625920.00	761200.82	453587.02	492129.77	448043.22
	Annual water temperature				
Mean	14.71	14.12	14.93	14.92	15.57

For both absolute fecundity ($p<0.05$ and $\chi^2 = 19.742 > \chi^2_critical = 9.49$, with $df = 4$ and $\alpha = 0.05$) and relative fecundity ($p<0.05$ and $\chi^2 = 57.857 > \chi^2_critical = 9.49$, with $df = 4$ and $\alpha = 0.05$), the Kruskal-Wallis tests indicated significant difference between years, meaning

that at least one year has a significantly different distribution compared to the others (Figure 3 a, b).

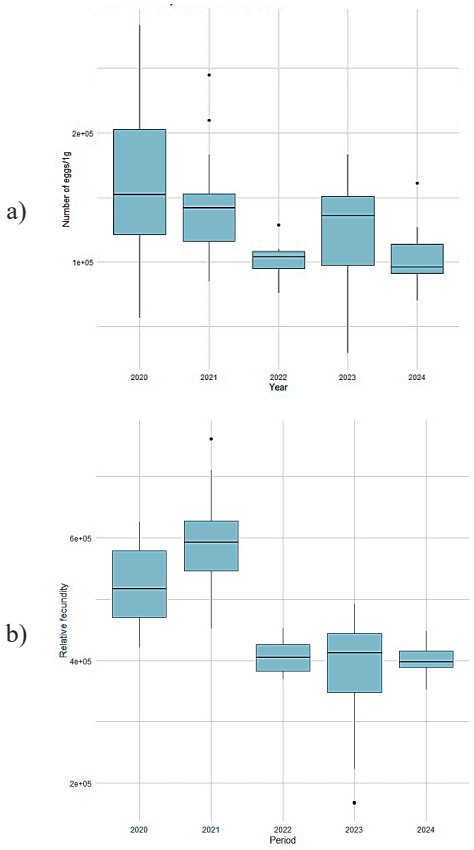


Figure 3 Variations between years in fecundity a) F; b) RF (Source: original)

The results show low values of absolute fecundity compared to those obtained by Raikova-Petrova et al. (2013), where the average fecundity reached up to 41,814 eggs in the 2010-2011 period.

The Dunn test, adjusted with the Bonferroni correction, showed significant differences between the absolute fecundity within the following years: 2020 vs. 2022 and 2020 vs. 2024 ($p<0.05$), suggesting that absolute fecundity was not constant across the years, indicating that ecological or biological changes may have influenced these values.

In case of RF, the variations were displayed differences between all years, but the most significant was 2021, when the relative fecundity was lower than the rest of the years.

There are two possible explanations that emerge. First, the egg production was lower than the rest of the years, which means that fish directed their energy more to growths and less to reproduction. Second, the individuals had a better body condition in 2021, thus reducing the RF value.

Regarding the GSI analysis, statistical tests revealed very significant differences (FS), especially between the years 2022-2023 and 2022-2024 ($\alpha = 0.99$) (Figure 4). These significant differences could indicate ecological, climatic, or biological changes that occurred during that period. The gonadosomatic index is a method used to measure the sexual maturity of the species in relation to the development of ovaries.

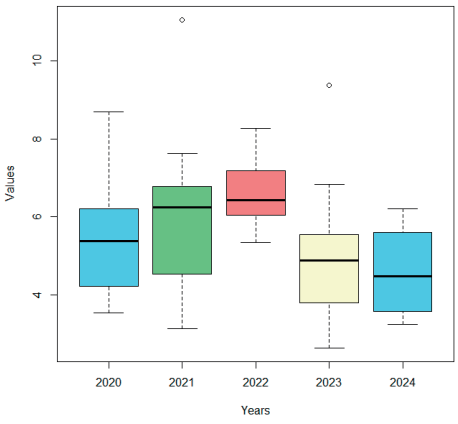


Figure 4. GSI Distribution per year for Pontic shad 2020-2024 (Source: original)

The trend in the average GSI values during this period was dome-shaped, with a maximum GSI of 6.65 reached in 2022 and a minimum GSI of 4.67 in 2024, which confirmed that relative fecundity decreased as the years progressed. The maximum GSI value in 2022 indicated that the species encountered favorable reproductive conditions that year. The minimum value per individual was 2.62, while the maximum was 11.06, values higher than those in 1987, which ranged from 2.61 to 9.14 (Năvodaru & Waldman, 2003).

Similar values were observed for the species *Alosa fallax* between 2022 and 2023 by Więcaszek et al. (2024) in the Szczecin Lagoon, Poland, another important spawning ground for the *Alosa* genus, like Chiscani, with

values ranging from approximately 128,000 to 158,000. As an anadromous species, *Alosa fallax* (Lacépède, 1800) exhibits a migratory behavior similar to that of the Pontic shad, traveling from marine environments to rivers or freshwater for spawning.

Correlating the RF with GSI (Pearson = 0.34), TW (Pearson = 0.10) and mean water temperature (Pearson = -0.81), resulted the existence of a strong negative influence of water temperatures that underlines the negative effect of temperatures variations on relative fecundity. As stated by Bowden (2008) and Rohlenová et al. (2011), the elevated temperatures can weaken the fish immune system, consuming energy reserves for survival, while other physiological tasks (e.g. growth, reproduction) are neglected.

Absolute Fecundity in relation to biological parameters

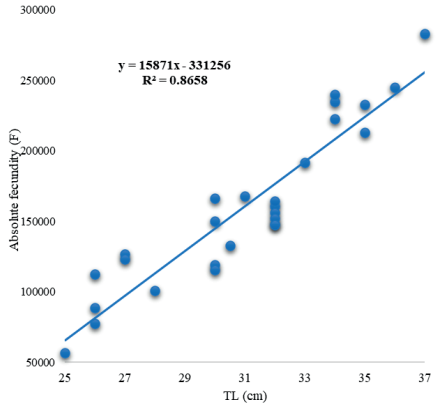
The 2020 exhibits an ascending trend in absolute fecundity in relation to total length (TL), age (t, years), and weight (TW) (Figure 5 a, b, c).

This trend is confirmed by the Pearson correlation coefficient, which reached significant positive values: 0.93 for Lt, 0.96 for age, and 0.95 for weight. These results suggest that as body parameters increase, the reproductive capacity of the individual intensifies. The coefficient of determination (R^2) of 0.8726 indicates that 87% of the variation in F can be explained by the variation in total length (TL). Additionally, for weight and age, the R^2 coefficients were 0.9074 and 0.9183, respectively, suggesting a significant influence of these parameters on absolute fecundity.

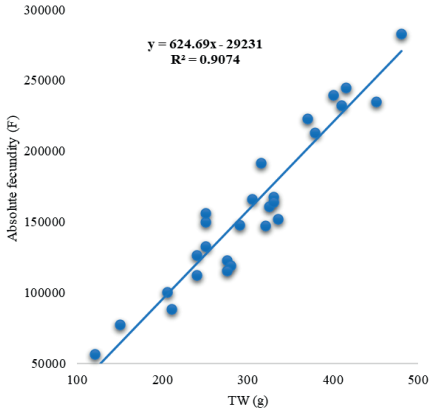
The determination relationships between F and body parameters are as follows:

- F = 15,871 * Lt – 33,1256;
- F = 624.69 * TW – 29,231;
- F = 50,800 * t – 27,707.

a)



b)



c)

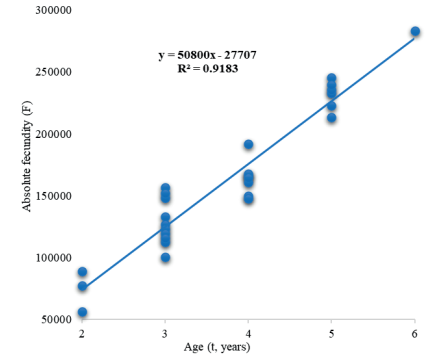
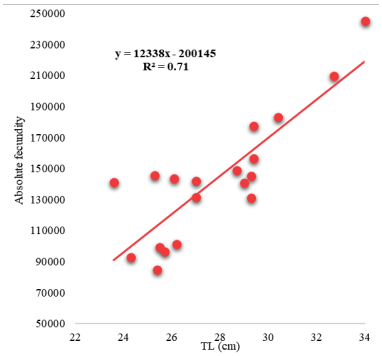


Figure 5. Absolute fecundity in relation to biological parameters for Pontic shad in 2020: a) F vs. TL; b) F vs. TW; c) F vs. Age (Source: original)

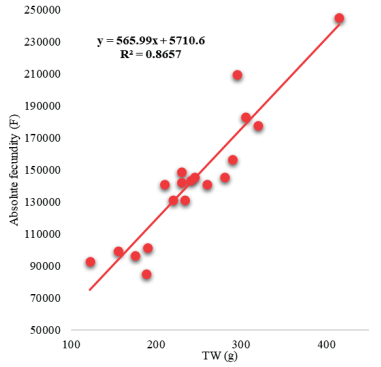
In 2021, the correlations between F and body parameters remain high, similar to the previous year (Figure 6).

The Pearson correlation coefficient reached 0.93 for weight (TW), 0.84 for total length (TL), and 0.96 for age (t).

a)



b)



c)

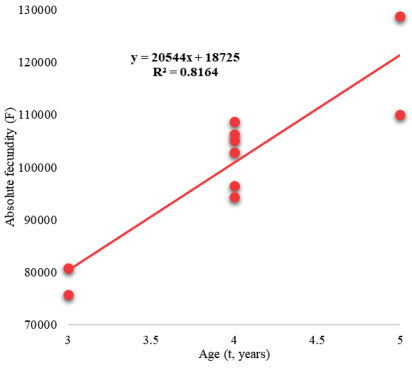


Figure 6. Absolute fecundity in relation to biological parameters for Pontic shad in 2021 a) F vs. TL; b) F vs. TW; c) F vs. Age (Source: original)

As shown in Figure 6 (a, b, c) and by the high values of the R^2 coefficient, age and weight are the best predictors of absolute fecundity, with R^2 values of 0.91 and 0.87, respectively. Although total length significantly influences fecundity, it has a weaker correlation compared to weight and age.

The models established for this year are:

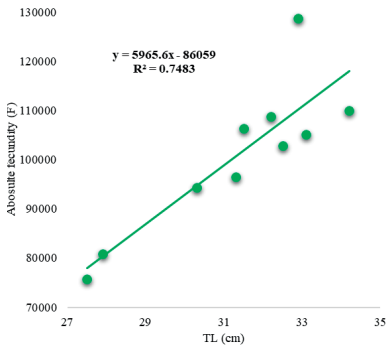
$F = 565.99 * TW - 5710.6;$

$F = 12338 * TL - 200145;$

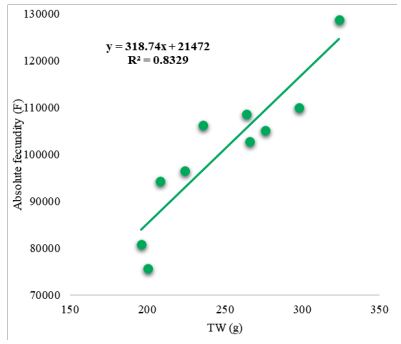
$F = 39027 * t - 13203.$

In 2022, the influence of body parameters on F remains significant. The Pearson coefficient is high for total length (0.86), age (0.90), and weight (0.91). Analyzing the variability of F, it is observed that 83.29% of its variation can be explained by weight variation, 74.83% depends on total length, and 81.64% on age (Figure 7 a, b, c).

a)



b)



c)

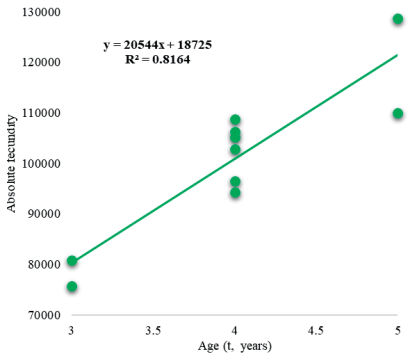


Figure 7. Absolute fecundity in relation to biological parameters for Pontic shad in 2022: a) F vs. TL; b) F vs. TW; c) F vs. Age (Source: original)

Additionally, the positive intercept suggests a minimum fecundity of approximately 21.472 units at lower weights.

The determination relationships are:
F = 318.74 * TW + 21472;
F = 5965.6 * TL – 86059;
F = 20544 * t + 18725.
In 2022 as well, weight (TW) and age (t) are the strongest predictors of absolute fecundity, suggesting that absolute fecundity increases with the size and age of the fish, which is characteristic of many oviparous fish species.
In 2023, the correlations between F and body parameters remain significant, with notable correlations for age, total length, and body weight (Figure 8 a, b, c). The determination relationships are:
F = 524.95 * TW – 39496;
F = 7232.2 * TL – 86927;
F = 40221 * t – 64763.

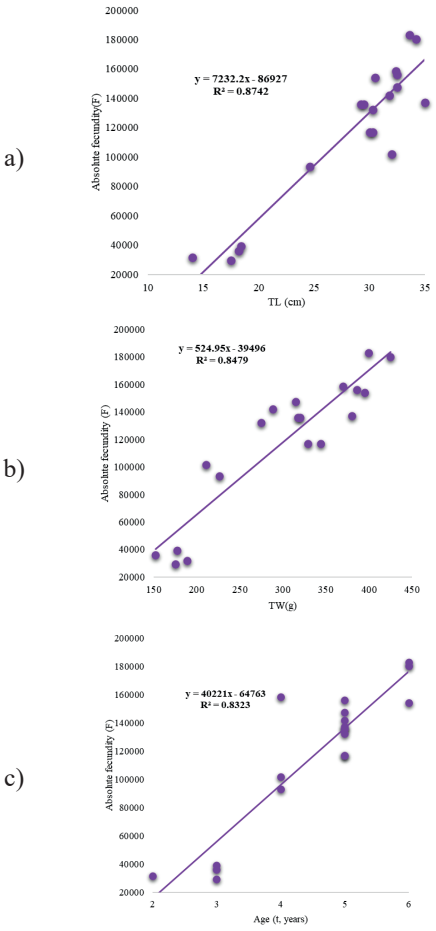


Figure 8. Absolute fecundity in relation to biological parameters for Pontic shad in 2023: a) F vs. TL; b) F vs. TW; c) F vs. Age (Source: original)

Additionally, all three relationships suggest that F increases as age, length, and body weight increase. Total length (TL) is strongly correlated with F (R^2 = 0.8742) and is considered the best predictor. Among these factors, total length (TL) has the strongest correlation with RF (R^2 = 0.8742), suggesting that body size is a better predictor for fecundity than age or weight.
The trend observed in 2023 continues in 2024, with strong relationships between F and body parameters (Figure 9 a, b, c). The coefficient of determination R^2 suggests that weight (TW) has the strongest correlation with F (R^2 = 0.918).
The relationships are:
F = 377.67 * TW + 5950.7;
F = 7187.8 * TL – 99730;
F = 23190 * t + 23769.

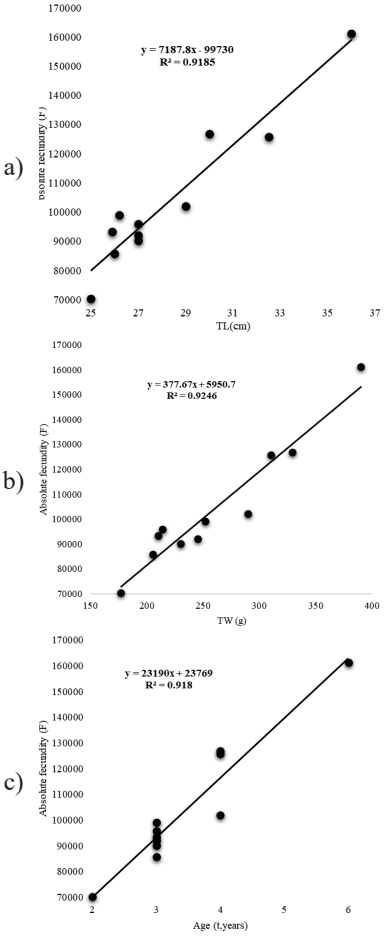


Figure 9. Absolute fecundity in relation to biological parameters for Pontic shad in 2024: a) F vs. TL; b) F vs. TW; c) F vs. Age (Source: original)

As Murua et al. (2003) stated, the study showed the relationship between the reproductive capacity of the shad specimens and body size, allowing for the estimation of how many eggs are produced by a specific stock of reproductive shad, considering factors such as size, age, and length of the fish. This was also confirmed by Raikova-Petrova et al. (2013) and Năvodaru & Waldman (2003). This is important for fishery resource management, as it enables the assessment of a population's ability to sustain and regenerate itself.

On the other hand, the results from Rozdina's (2015) study on the shad in the Bulgarian Danube area suggested an inverse relationship between absolute individual fecundity and the fish's weight, meaning that fecundity decreases as the linear growth rate and weight of the fish increase.

CONCLUSIONS

The study highlighted a significant correlation between biological parameters, such as weight, length, and age, and the relative fecundity of the Danube shad. These correlations suggest that body size and age are important predictors of reproductive capacity, confirming trends observed for many oviparous fish species. Thus, evaluating these parameters can provide valuable information for managing and conserving fishery resources.

The predominance of females in shad populations could indicate a stressed population, but it may also be the result of fluctuating cohort strength and different ages at maturation between males and females. An imbalanced sex ratio can influence reproductive processes and may suggest higher selective pressure on males. This imbalance could affect the long-term structure of the population, with potential implications for its stability.

Data collected for the years 2020-2024 suggest a downward trend in absolute fecundity, which may reflect ecological, biological, or environmental changes that have affected the reproductive capacity of Danube shad. It is essential to monitor these changes to identify the causes behind this decrease and to take appropriate actions.

In light of the decrease in absolute fecundity observed in recent decades, it is recommended to study the causes behind this decline. Comparing current data with historical records will help identify the extent to which temperature fluctuations contribute to this trend. Understanding the biological, ecological, and environmental drivers behind these changes is crucial for developing effective conservation and sustainable management strategies for shad populations.

ACKNOWLEDGEMENTS

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HARNESSING GREEN MACROALGAE FOR SUSTAINABLE FISH FEED: OPPORTUNITIES AND CHALLENGES

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Abstract

The world faces a critical challenge in ensuring sufficient food production to meet the needs of a growing global population while maintaining nutritional quality and promoting environmental sustainability. Among sustainable food sources, seaweed, particularly the genus Ulva, has emerged as a promising solution due to its nutritional composition, abundance, and accessibility. However, integrating Ulva into animal feeds presents challenges, including nutritional value variability and indigestible polysaccharides, which reduce energy availability. This review explores the potential of Ulva sp. as an ecological and nutritious ingredient for aquaculture. It highlights the need for optimized nutritional strategies and processing technologies to increase protein content and improve nutrient digestibility. The actual status of the biochemical composition of Ulva and its benefits in commercial fish feed are emphasized, focusing on fish growth performance, stress resistance, immune function, and gut microbiota health. Drawing on over 50 studies, the review underscores positive trends. It identifies optimal inclusion levels for Ulva in fish diets, aiming to enhance digestibility and functional properties while addressing sustainability goals in aquaculture.

Key words*: functional compounds, nutritional, Ulva.*

INTRODUCTION

By 2050, population growth and climate change will exert considerable pressure on global food security, requiring sustainable solutions (Molotoks et al., 2021). Aquaculture plays a key role in this context, providing a sustainable alternative for protein supply and supporting responsible economic development. The integration of ecological principles in this industry contributes to the protection of aquatic ecosystems and the conservation of natural resources, reducing the impact on the terrestrial environment (Ahmed et al., 2019; Choudhary et al., 2021).

Aquaculture has emerged as one of the fastest-growing sectors in food production, providing an essential source of protein (Anderson et al., 2017). As this sector develops, the demand for feed has increased significantly, highlighting the need to identify alternative protein sources amid the declining availability of animal-based options (Aragão et al., 2022). Marine algae offer a promising solution due to their high nutritional value and environmental benefits, making them a viable alternative to conventional feed ingredients (Pereira et al., 2024). A well-balanced diet is crucial for ensuring the optimal health, growth, and productivity of farmed fish. However, the aquaculture industry's heavy reliance on fishmeal as the primary protein source raises significant sustainability concerns. These challenges stem from the depletion of wild fish stocks, the rising global demand for fishmeal, and the escalating costs associated with its production and procurement (Jannathulla et al., 2019). As a result, researchers and industry stakeholders are actively exploring alternative, more sustainable protein sources, to reduce dependence on fishmeal while maintaining fish health and performance.

Green algae, such as *Ulva lactuca*, stand out for their rich nutrient profile and bioactive compound content, offering significant potential for the development of functional diets for aquaculture fish (Holdt & Kraan, 2011).

Recent studies highlight that functional metabolites of seaweeds not only support growth performance but also enhance immunity, boost antioxidant status, and increase disease resistance, thus contributing to the maintenance of fish health (Aragão et al., 2022; Mota et al., 2023). However, there have been studies indicating that high levels of inclusion could lead to less efficient utilization of feed due to limited digestibility and nutrient uptake, which would negatively affect the growth rate (Wan et al., 2019; Pratiwi & Pratiwy, 2022).

In this context, the present study analyzes the nutritional properties and bioactive compounds found in seaweed, emphasizing their potential benefits for the growth, health, and overall welfare of farmed fish. Additionally, the paper explores strategies for improving seaweed processing methods to enhance its nutritional value while minimizing any potential adverse effects associated with its inclusion in aquaculture feed.

MATERIALS AND METHODS

This review comprises literature obtained from recognized academic databases such as Web of Science, Scopus, PubMed and Google Scholar.

The search strategy incorporated Boolean operators (AND, OR) and MeSH terms to refine results. Keywords and phrases used included: “green macroalgae”, “fish nutrition”, “growth performance”. “feed utilization”, “bioactive compounds”, “Ulva in aquaculture”, “immunostimulants”, “marine-derived polysaccharides”.

The selection of studies was based on predefined inclusion and exclusion criteria to ensure the relevance and quality of the data analyzed. Studies were included if they were published in peer-reviewed journals within the last two decades and provided experimental research, systematic reviews, or meta-analyses related to the utilization of macroalgae in aquafeeds. Only studies that contained information on nutritional composition, bioactive compounds, fish growth performance, immune response, or processing techniques for improving macroalgae digestibility were considered. In contrast, studies were excluded if they lacked experimental validation, were limited to theoretical discussions, or focused solely on microalgae or other non-macroalgal marine resources. Additionally, research with insufficient sample size or statistical significance was omitted.

RESULTS AND DISCUSSIONS

Optimizing production of macroalgae

Throughout history, macroalgae have been prized for their nutritional value, and are still part of the Asian diet today for their multiple health benefits (Hafting et al., 2015). However, with the intensification of studies on the macroalgae composition and on their bioactive compounds, the exploitation has registered a significant expansion globally (Dominguez & Loret, 2019; Kidgell et al., 2019). In this context, to ensure sustainable production, the optimization of harvesting and cultivation methods is essential for enhancing production of bioactive compounds. The availability and nutritional composition of Ulva biomass in natural stocks are influenced by a range of dynamic factors, including species and strain diversity, environmental conditions, associated microbiome, geographical location, and seasonal variations (Table 1). These fluctuations present significant challenges in ensuring the consistent quality and optimal utilization of Ulva as a sustainable resource for aquaculture and other applications.

Table.1 Nutritional composition of various species of Ulva

Species	Carbohydrates (%dw)	Proteins (%dw)	Lipids (%dw)	Ash (%dw)	References
Ulva lactuca	58.1	13.6	0.19	11.2	(Rasyid, 2017)
Ulva lactuca	54.95 ±1.43	14.58±1,30	0.69±0.06	18.38±3.08	(Sirbu et al., 2020)
Ulva lactuca	61.83 ± 0.01	10.0 ± 0.01	0.13 ± 0.01	17.86 ± 0.87	(Pangestuti et al., 2021)
Ulva rigida	31.87 ± 0.26	27.11 ± 0.62	2.71 ± 0.70	19.63 ± 0.63	(Kumar et al., 2021)
Ulva rigida	27.9±0.4	19.5±0.1	0.08±0.004	26.6±0.4	(Nova et al., 2023)
Ulva fasciata	545.301	38.897	0.1878	204.842	(Anis et al., 2018)
Ulva fasciata	32.0 ±0.04	22.7 ± 0.22	0.89 ± 0.12	27.0 ± 0.024	(Ganesan et al., 2020)

Species	Carbohydrates (%dw)	Proteins (%dw)	Lipids (%dw)	Ash (%dw)	References
Ulva reticulata	46.9 ± 0.56	9.86 ± 0.67	0.28 ±0.45	2.70 ± 0.32	(Jayasinghet et al., 2019)
Ulva reticulata	48.40±0.0	12.93±0.08	3.04±0.07	23.32±0.02	(Djoh et al., 2024)
Ulva intestinalis	57.03 ± 1.36	13.55 ± 0.07	2.72 ± 0.28	19.01 ± 1.15	(Jannat-Alipour et al., 2019)
Ulva intestinalis	5.16 ± 0.04	3.32 ± 0.14	0.04±0.01	5.62 ± 3.20	(Farzanah et al., 2022)
Ulva pertusa	-	15.4 ± 0.9	4.8 ± 2.9	27.2 ± 1.7	(Benjama & Masniyom,2011)
Ulva pertusa	52.3	25.1	0.1	22.5	(Lee et al., 2014)
Ulva clathrata	-	20.1 ± 0.1	2.2 ± 0.1	27.5 ± 0.2	(Peña-Rodríguez et al., 2011)

These factors directly impact both the yield and the overall availability of Ulva resources (Simon et al., 2022). Consequently, sustainable harvesting practices are crucial to prevent overexploitation and reduce environmental impact. However, some studies suggest that natural Ulva stocks may not be sufficiently productive for large-scale commercial harvesting, and the seasonal fluctuations in biomass make it challenging to accurately estimate production, ultimately affecting the ability to meet commercial demands (Calheiros et al., 2021).

Algaculture presents a viable and sustainable alternative for meeting industrial algae demands, particularly when natural harvesting becomes insufficient due to low biomass availability or inconsistent quality (Yong et al., 2022). By providing controlled cultivation conditions, algaculture ensures a more reliable and scalable supply of high-quality Ulva biomass, reducing dependence on wild stocks and supporting long-term sustainability.

To ensure a sustainable and environmentally friendly source of biomass, the macroalgae Ulva are cultivated in various types of systems. Seaweed culture systems can be realized offshore (open sea method), on land (onshore), where conditions are controlled, or close to the coast (nearshore), the latter being the most widely used aquaculture technique in estuaries and coastal areas (García-Poza et al., 2020). Beyond the simplistic approach of seaweed culture as a single production, seaweed can be integrated into a multitrophic aquaculture system (IMTA). *Ulva lactuca* cultivated in this system maintains a stable nutritional profile throughout the year, ensuring constant quality (Hayashi et al., 2014; Marinho-Soriano, 2017; Laramore et al., 2022).

Establishing an ideal protocol for obtaining the best Ulva biomass is a complex challenge, as each aquaculture system operates under specific environmental conditions, which directly influence the final result.

The correct classification of Ulva biomass is essential, given that about 24-32% of Ulva species are incorrectly identified in genetic databases (Fort et al., 2022), the errors being caused either by phenotypic variations influenced by environmental factors (Wolf et al., 2012) or by specific differences between the morphological and genetic traits of the species (Fort et al., 2019; Olsson et al., 2020; Toth et al., 2020; Cardoso et al., 2023). Thus, genetic improvement of seaweed can play a key role in optimizing the long-term viability, growth, and sustainability of global cultivation industries, providing significant benefits in this sector (Robinson et al., 2013).

Ulva - an alternative to fishmeal

The ratio of essential and non-essential amino acids, similar to that of soybean meal (Shuuluka et al., 2013), together with balanced protein content, comparable to that of plants (Stedt et al., 2022), are key arguments for the use of green macroalgae as alternative protein sources in fish feed. Although green seaweed has a low lipid content, it is important to note that the lipid fraction contains high levels of essential polyunsaturated fatty acids, such as linoleic acid and α-linolenic acid (Aguilera-Morales et al., 2018), which are essential for both human and fish nutrition (Zárate et al., 2017; Galindo et al., 2021).

Furthermore, saturated fatty acids like palmitic acid, which some researchers have identified as the primary component (Ortiz et al., 2006; Horincar et al., 2014), along with a low omega-6 to omega-3 fatty acid ratio, indicate that green algae may serve as a valuable food source or dietary supplement with the potential to address omega-3 deficiencies (Schmid et al., 2014; Sohrabipour, 2019). At the same time, they, are distinguished from conventional

plant-based foods by their high content of minerals, such as magnesium, calcium, iron, selenium, sodium, and potassium, as well as the presence of essential vitamins, such as B12 and C, along with lipophilic vitamins, such as A and E (tocopherol) (Jacobsen et al., 2023).

Since 1984, with the first research on the use of Ulva extract in fish diets (Nakagawa et al., 1984), scientific interest in this macroalga has increased significantly. Subsequent studies investigated the effects of different species of the genus Ulva on various fish species, highlighting the potential of this resource in fish nutrition. The selection of Ulva species such as *Ulva lactuca*, *Ulva rigida*, *Ulva pertusa*, *Ulva ohnoi* and *Ulva intestinalis* was probably mainly influenced by their accessibility in the wild and their nutritional profile. Most of the studies assessed Ulva spp. coming from the wild environment (Ortiz et al., 2006; Azaza et al., 2008; Yildirim et al., 2009; Natify et al., 2009; Abdel Aziz & Ragab, 2017; Valente et al., 2016; Tapia-Paniagua et al., 2019) rather than integrated multitrophic aquaculture (IMTA) due to the low number of experiments carried out (Neori et al., 2000; Schuenhoff et al., 2003; Valente et al., 2006; Marinho et al., 2013; DM Silva et al., 2015; Shpigel et al., 2017). The partial substitution of fishmeal with green macroalgae meal has been extensively analysed, considering both the method of incorporation and the optimal inclusion levels in aquafeeds. A review of post-2015 literature reveals two primary approaches for utilizing *Ulva* spp. in fish nutrition: the direct use of raw biomass (either fresh or dried) and the integration of protein extracts obtained through advanced processing techniques, aiming to enhance digestibility and nutritional efficiency. Thus, macroalgae supplementation in the form of whole biomass (fresh or dry) ranged from 2.6% to 100% (Table 2), while integration in the form of extract ranged from 0.1% and 3% (Table 3). This variation is dependent on both the fish species and the type of Ulva spp. used in the experiments. Reported results indicated that a moderate replacement of fishmeal with Ulva can have a positive impact on the dietary intake and development of fish, due to the high nutritional value of this macroalgae. However, excessive substitution had negative effects, such as poor development and inefficient feed utilization, which reduced palatability of the diet due to the presence of anti-nutritional factors.

Scientific research has shown that the incorporation of whole macroalgae in aquaculture diets has primarily been studied in relation to growth performance, feed utilization efficiency, and body composition. In contrast, studies focusing on the use of macroalgae extracts have concentrated on evaluating their impact on health indicators, particularly immunological response and antioxidant activity, to assess potential benefits for fish health and disease resistance (Figure 1).

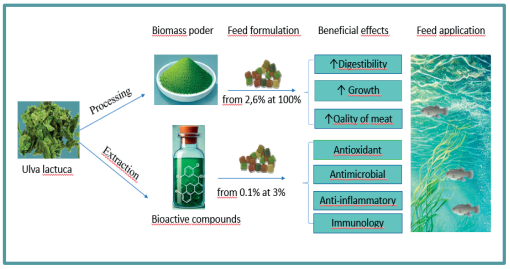


Figure 1. Overview of the use of green macroalgae in the diet of aquatic organisms and their nutritional benefits (after Gonzalez et al., 2023)

The effect of *Ulva* sp. on the fish growth

Studies focusing on the incorporation of macroalgae, either as fresh or dried biomass, in the diets of aquatic organisms has primarily focused on assessing growth performance, feed utilization efficiency, and body composition (Table 2). The integration of whole algal biomass at a 5% inclusion rate has produced inconsistent results, underscoring the need to further optimize both proportions and conditions. Thus, some research reports slower development and inefficient feed utilization for *Solea senegalensis* (Fumanal et al., 2020; Tapia-Paniagua et al., 2019), while others highlight positive growth effects on species like *Luthanus stellatus* and *Cirrhinus mrigala* (Zhu et al., 2016; Upreti et al., 2021). At the same time, Mandibana et al. (2017) identified 5% as the upper inclusion limit for Ulva macroalgae, showing that this concentration maintains both growth parameters and the health of *Argyrosomus japonicus* juveniles. Moreover, a study by Valente et al. (2016) demonstrated that including 5% Ulva spp. in the diet of *Oreochromis niloticus* led to a significant

increase in carotenoid accumulation in the skin. Regarding the 10% inclusion level, studies showed improved growth and feed utilization efficiency, compared to control diets, for species like *Oreochromis niloticus* (Suryaningrum & Samsudin, 2020) or *Scatophagus argus (*Yangthong & Ruensirik, 2020). In contrast, the inclusion of *Ulva lactuca* and *Ulva rigida* in the diets of *Oreochromis niloticus* (Valente et al., 2016) and *Clarias gariepinus* (Abdel-Warith et al., 2016) is sustainable up to a 10% threshold, without negatively impacting growth parameters. Other authors (Elsharkawy et al., 2022) observed significant improvements in growth performance and feed utilization, as well as enhanced physiological responses in tilapia when replacing soybean meal with Ulva flour at a moderate 15% level. Similarly, replacing fishmeal with 14.6% *Ulva lactuca* from an Integrated Multi-Trophic Aquaculture (IMTA) system and 14.1% poultry flour resulted in performance comparable to the control diet for *Sparus aurata* (Shpigel et al., 2017).

On the other hand, higher levels of inclusion - 50% fresh *Enteromorpha* macroalgae mixed with 50% artificial feed- generated optimal performance for the herbivorous fish such as *Siganus rivulatus*. Instead, the complete replacement of artificial feed with fresh macroalgae harmed growth (Abdel Aziz & Ragab, 2017).

Castellon (2019) observed growth performance improvements in *Girella laevifrons* when supplemented with 30% Ulva, although these advances were associated with a reduced survival rate, likely due to the effects of handling or manipulation. Similarly, Younis et al. (2019) found that the inclusion of *Ulva lactuca* flour at levels of up to 20% in the diet of *Oreochromis niloticus* resulted in significant growth improvements, without any adverse effects on growth parameters or feed utilization efficiency.

Table 2. Utilization of macroalgae as whole biomass, either fresh or dried, in the fish diet

***Ulva* species**	**Fish species**	**Inclusion of *Ulva* spp.(%)**	**Effects on growth**	**Reference**
Ulva lactuca	*Girella laevifrons*	15, 30, 45	30%↑WG,low survival	(Castellón, 2019)
Ulva lactuca	*Oreochromis niloticus*	20, 40, 60	20%↑WG,SGR 60%↑carcass protein level	(Younis et al., 2019)
Ulva lactuca	*Oreochromis niloticus*	5, 15, 20, 35	15%↑WG,SGR ↓FCR 35%↑ carcass protein level	(Elsharkawy et al., 2022)
Ulva lactuca	*Sparus aurata*	2.6 & 7.8(exp1) 14.6 & 29.1(exp2)	↔WG,SGR at 14.6 ↓WG,SGR at 29.1	(Shpigel et al., 2017)
Ulva fasciata Enteromorpha	*Siganus rivulatus*	50, 100	50%↑WG,SGR↓FCR ↑lipid content in body	(Abdel Aziz & Ragab, 2017)
Ulva lactuca	*Argyrosomus japonicus*	50, 100, 150, 200 gKg^{-1}	50gKg↑WG↓SGR	(Madibana et al., 2017)
Ulva ohnoi	*Solea senegalensis*	5	5%↓WG,↔FCR	(Fumanal et al., 2020)
Ulva ohnoi	*Solea senegalensis*	5	5%↓WG,SGR↑FCR	(Tapia-Paniagua et al., 2019)
Ulva lactuca	*Luthanus stellatus*	5, 10, 15, 20	5%↑WG,SGR↓FCR,and ↓ protein and lipid content in the whole body	(Zhu et al., 2016)
Ulva meal	*Cirrhinus mrigala*	5, 10, 15	5%↑WG,SGR↓FCR	(Upreti et al., 2021)
Ulva rigida Ulva lactuca	*Oreochromis niloticus*	5, 10	5,10%↔WG,SGR,FCR 5%↑total carotenoids in skin	(Valente et al., 2016)
Ulva lactuca	*Clarias gariepinus*	10, 20, 30	10%↑WG,SGR 30%↑ protein in the muscles	(Abdel-Warith et al.2016
Ulva meal	*Oreochromis niloticus*	10, 20, 30	10%↑WG,SGR ↑body protein level	(Suryaningrum & Samsudin, 2020)
Ulva Lactuca	*Scatophagus argus*	5, 10, 15, 20, 25, 30	10%↑WG,SGR	(Yangthong & Ruensirikul,2020)

WG - weight gain; FCR - feed conversion ratio; SGR - specific growth rate; ↑ increase; ↔ no change, ↓ decrease compared to the control diet ($P < 0.05$)

Impact of *Ulva* spp. extract on antioxidant status and immune system of fish

Recent research has highlighted the potential of green macroalgae not only as a food protein source, but also as functional ingredients in diets intended for various fish species. Studies have examined the integration of green macroalgae extracts, focusing in particular on effects on oxidative status, immune response modulation, intestinal and microbiota analysis (Table 3).

Table 3. Utilization of macroalgae extracts in the fish diet

Extract seaweed	Fish species	Inclusion %	Health effects	References
Ulvan	*Oreochromis niloticus*	0.1%, 0.5% and 1%	↑ immune response	del Rocio Quezada & Fajer Avila, 2017
Polysaccharide Ulva (WPU)	*Mugil cephalus*	0.5, 10 and 15 mg kg^{-1}	WPU 10 ↑antioxidant activity ↑immune responses	Akbary & Aminikhoei, 2018
Ulvan	*Labeo rohita*	0, 25, 50 and 100 mg/kg	50 mg/kg^{-1}↑growth, ↑lysozyme activity ↑protection against F.columnare	Harikrishnan et al., 2021
Extract of Ulva/Gracilaria	*Oncorhynchus mykiss*	0.5 gkg^{-1},1.5 gkg^{-1}SPU 0.5 gkg^{-1},1.5 gkg^{-1}SPG	1.5 gkg^{-1}SPU/SPG↑growth, ↑lyzozyme activity,↑ACH50	Safavi et al., 2019
Extract of Ulva	*Chanos chanos*	0, 100, 200, 300 400 and 500 ppm	500 ppm↑growth	Nurfadillah et al., 2021
Ulva fasciata /metanol extract (UFME)	*Oreochromis niloticus*	0, 50, 100 and 150 mg kg^{-1} UFME	100 $mgkg^{-1}$↑ the length of intestinal villi,↑immunity capacity	Abo et al., 2021
Seaweed liquid extract TAM	*Oreochromis niloticus*	0.5, 1, 1.5 and 2%	2%↑growth,↑ feed utilization,↑ lysozyme activity and respiratory burst assay	Ashour et al., 2020
Seaweed mixture SME	*Pangasianodon hypophthalmus*	1, 2 and 3%	2 and 3%↑growth, ↑antioxidant status and immune biomarkers.	Abdelhamid et al., 2021
Polysaccharide Alginium(AL)	*Sparus aurata*	(3g/kg)-AL0.3 and (5g/kg) -AL0.5	↑ growth,↑innate and adaptive immune responses,↑ resistance against *Photobacterium damselae*	Güroy et al., 2022

Nevertheless, some of these studies, beside health issues, addresses also growth performance.

For instance, a study conducted on *Oreochromis niloticus* evaluated the effects of different Ulvan concentrations (from 0.1% up to 1%). The findings indicated that while supplementation did not significantly impact growth performance, it demonstrated a notable immunomodulatory potential, with its activity being sustained even after supplementation was discontinued (del Rocio Quezada-Rodriguez & Fajer-Avila, 2017). Similarly, supplementing the diet of *Labeo rohita* with 50 mg/kg of ulvan led to enhanced growth rates, increased serum lysozyme enzyme activity, and improved immune responses (Harikrishnan et al., 2021). Moreover, by modulating the expression of immuno-antioxidant genes such as GPx, CAT, and SOD, ulvan supplementation conferred protection against bacterial infection caused by *Flavobacterium columnare*.

In another study, Akbary & Aminikhoei (2018) investigated the dietary supplementation of water-soluble polysaccharides from *Ulva rigida*, particularly at the WPU 10 level, and observed a significant enhancement in lysozyme activity and phagocytic processes, contributing to an increase in final body mass. Additionally, supplementation resulted in an increase in total antioxidant content (TAC), superoxide dismutase (SOD) activity, and reduced glutathione (GSH) levels, while simultaneously leading to a considerable decrease in malondialdehyde (MDA) levels in liver tissue, indicating a reduction in oxidative stress. Mortality was significantly reduced compared to the control group, and the WPU 10 dose was found to be most effective for protecting *Mugil cephalus* against *Photobacterium damselae* infection. Another author (Safavi et al., 2019) observed that the effect of sulfated polysaccharide extract of green alga *Ulva*

intestinalis (SPU) and red alga *Gracilariopsis persica* (SPG) produced a significant increase in growth performance, and lysozyme and serum complement activity in fish-fed 1.5% SPG and 1.5% SPU. Also, in these experimental groups, superoxide dismutase activity was lower compared to the control group.

Similarly, Abo et al. (2021) investigated the effect of supplementing the diet with methanolic extract of *Ulva fasciata* on the species *Oreochromis niloticus*, finding that a concentration of 100 mg/kg resulted in significant increases in final weight. The trial emphasized significant improvements in the length of intestinal villi, which was considerably increased in all segments of the intestine. The enzymatic activity of SOD and CAT was also significantly higher ($p < 0.05$). In addition, there was an increase in lysozyme activity and phagocytic activity, indicating an improvement in immune mechanisms. Nurfadillah et al. (2021) also observed that the diet containing ethanolic extract at a concentration of 500 ppm resulted in the best growth performance for *Chanos chanos*. Other studies demonstrated that adding up to 2% of the commercial seaweed supplement TrueAlgaeMax (TAM) to the diet of *Oreochromis niloticus* optimizes growth, feed efficiency and nonspecific immunity against *Aeromonas hydrophila* infections (Ashour et al., 2020), while adding an extract from a mixture of seaweed in proportions of 2-3% it improves the antioxidant status, highlighting the protective effect on the liver of fish, and supports both innate immunity and growth performance (Abdelhamid et al., 2021).

Güroy et al. (2022) pointed out the potential use of Algimun® - a mixture of sulfated marine polysaccharides in the *Sparus aurata* diet, demonstrating that supplementation with this additive significantly improves growth performance, reduces the rate of food conversion, and stimulates both innate and adaptive immune responses. It was also found to increase fish resistance and survival from *Photobacterium damselae*.

Strategies for macroalgae integration in fish nutrition

The marine macroalgae industry is well consolidated and contributes about 30% to global aquaculture production. Annually, it generates a volume of about 30 million tons, with an estimated value of over 6 billion dollars. The main producing countries are China and Indonesia, which contribute about 90% of total production (Hua et al., 2019). In Europe, the macroalgae industry continues to be dominated by harvesting from natural ecosystems, which provides 68% of the total production units. However, macroalgae aquaculture, carried out in both terrestrial and marine systems, is in full development in several European countries, reaching to cover 32% of the production capacity. Norway stands out as the main producer of marine algae biomass in Europe (Araújo et al., 2021). Ulva spp. are not among the most intensively cultivated species in aquaculture. The main producers of green algae *Ulva* spp. are South Africa, with a production of 3,175 tons in 2020, and China, with a volume of 200 tons in the same year (Hofmann et al., 2024). In Europe, production data was not fully reported to the FAO, only information provided by Portugal and Spain is available. The economic impact of using this macroalgae in the fish diet remains poorly documented.

Shpigel et al. (2017) were the first to estimate the economic benefits associated with the use of Ulva in an integrated multi-trophic aquaculture system (IMTA), highlighting the impact on feed costs, fish production, and operational expenditure. Given that fish feed may account for more than 60% of total costs in intensive aquaculture, a reduction of around 10% of these expenses is of significant economic importance.

Challenges associated with NSPs in macroalgae

Due to their rich nutritional composition, macroalgae are regarded as a promising ingredient for aquafeeds. However, their widespread utilization is constrained by the presence of antinutritional factors, particularly non-starch polysaccharides (NSPs) such as cellulose, galactans, xylans, and hemicellulose, which can negatively impact digestibility and nutrient absorption. When included in high proportions, these compounds reduce feed palatability, hinder the efficient utilization of nutrients, and ultimately impair growth performance (Abdel-Warith et al., 2016;

Moutinho et al., 2018; Tapia-Paniagua et al., 2019). In this context, the use of enzymes as feed additives for fish was investigated to evaluate their potential effects as a means of improving the efficiency of the feed and, implicitly, the digestibility of macroalgae proteins (Castillo & Gatlin, 2015; Xie et al., 2019). All diets have been accepted by fish, and those with enzymes have shown beneficial effects on growth and digestion, without significant differences from the control diet. This suggests that enzymes could reduce the disadvantages of macroalgae-based diets. Similarly, the study conducted by El-Mousalamy et al. (2022) investigated the feeding of *Oreochromis niloticus* to evaluate the effects of supplementing *Ulva lactuca* - based diets with exogenous enzymes, including a monoenzymatic and multienzymatic complex (MEM), as well as yeast. A fishmeal-based (FM) diet was used as a positive control. The results indicated that the diet supplemented with the multi enzymatic complex (MEM) achieved the highest growth parameter values, with no significant differences between this treatment and the control dietThe feed conversion coefficient (FCR) values were significantly lower in fish fed on the fish meal (FM) diet and on diets containing Ulva supplemented with enzymes or yeast. The increased feed conversion efficiency and palatability of the diet can be attributed to the more effective release of nutrients from plant diets, due to the degradation of antinutritional factors (ANF). These processes favored both the development of protein digestion and more efficient absorption of amino acids (Maryam et al., 2024)

In addition to the advantages of enzyme supplementation, the use of probiotics as additives in the diet presents promising prospects, providing multiple benefits to the hosts. They contribute to the production of digestive enzymes, stimulating the intestinal microflora and thus enhancing nutrient intake and intensifying fish metabolism (Zamini et al., 2014; Adeoye et al., 2016). This aspect is also highlighted in the study conducted by Amer et al. (2020), which evaluated the impact of using fermented lactic algae (FER) and exogenous enzyme blends supplemented with Natuzyme®, administered in combination with L-carnitine (LC) and/or probiotics (PRO) in the diet of *Oreochromis niloticus*. Additionally, the research conducted by Tharaka et al. (2020) analyzed the effects of supplementing the diet of *Paralichthys olivaceus* with algal clay powder, derived from *Ulva lactuca* and *Solieria chordalis*, integrated into exfoliated micronized montmorillonite (ACP). The results indicated that a fishmeal-deficient diet supplemented with 0.2% algal clay powder had beneficial effects on growth parameters and feed utilization efficiency. These improvements were attributed to the presence of bioactive compounds in the algae and the optimization of intestinal morphology due to montmorillonite (Nur et al., 2020; Karimi et al., 2020)

Another similar study conducted by Gunathilaka et al. (2021) investigated the effect of dietary supplementation with marine algae extracts (*Ulva* spp. and *Solieria* spp.), both in the presence and absence of organic acids, on the species *Paralichthys olivaceus*. The results indicated that dietary supplementation had a beneficial impact on intestinal morphology and digestive enzyme activity. Increased activity of digestive enzymes has also been reported in other studies conducted on various fish species such as *Sciaenops ocellatus* and *Oreochromis niloticus* fed diets enriched with organic acids (Castillo et al., 2014; Addam et al., 2019).

Antinutrients in macroalgae: implications for fish nutrition

The bioactive compounds in macroalgae are essential for maintaining health, helping to increase nutrient absorption, creating gut microbiota, and supporting immune function. Although they bring numerous nutritional benefits, it is important to consider the presence of antinutrients in their biochemical composition. Among the antinutrients found in macroalgae, the most common are: tannins, phytates, oxalates, saponins, lectins, and alkaloids (Francis et al., 2001; Gemede, 2014). These natural compounds can affect the absorption and use of nutrients, thus having a significant impact on fish health. To mitigate the adverse effects of nutrients, various processing methods can be used, such as fermentation and thermal and enzymatic treatments. These techniques can reduce the levels of antinutrients and increase the

nutritional values of macroalgae. On the other hand, macroalgae extracts are a promising alternative that offers higher concentrations of specific bioactive compounds thus providing potential additional benefits.

The thermal processing of macroalgae can be carried out for various purposes, including inactivation of associated microorganisms, thus facilitating their use in human nutrition. Also, this process can help create the nutritional value of macroalgae, making them more suitable as additives in the feed of aquatic organisms. The effect of thermal processing on the macroalgae *Ulva hanoi* was investigated in a 90-day feeding study conducted on *Solea senegalensis* (Vizcaino et al., 2019). In this study, the diet enriched with 5% Ulva was processed through a four-section extruder, applying a progressive thermal profile: 100°C at the beginning, followed by 95°C, 90°C, and finally 85°C at the exit. Experiments have demonstrated good food acceptability without a significant impact on food consumption. However, the temperature applied during extrusion was not sufficient to fully inactivate the protease inhibitors present in the macroalgae. Nevertheless, analysis of the ultra-structural morphology of the intestinal mucosa showed an increase in the absorption surface after 45 days of administration, an effect that was not maintained until the end of the experimental period**.**

Similarly, other authors (Fernandes et al., 2022) evaluated the impact of innovative treatments on the composition and structure of the macroalga *Ulva rigida*, aiming to integrate it into the diets of aquatic organisms, using *Dicentrarchus labrax* as an experimental model. In this study, six experimental variants were tested, in which 5% of the feed ingredients were replaced with treated *Ulva rigida* macroalgae using various methods: alkaline, autoclave, ultrasound, microwave, liquid fermentation with microwave (SSF), and solid fermentation followed by sequential hydrolysis (SSF-SH). Of these, SSF-SH caused the greatest degradation of cellulose and hemicellulose, as well as the release of the highest amounts of reducing sugars. Although fish have accepted all experimental diets, *Ulva rigida* supplementation, regardless of treatment, reduced voluntary food consumption and significantly affected the growth performance compared to the positive control diet. The only exception was represented by the variant treated by SSF, which sustained similar growth to that seen in the positive control group.

In contrast, a study conducted by Magnoni et al. (2017) on the species *Sparus aurata* examined the effects of supplementing the diet with 5% thermal-treated marine algae *Gracilaria vermiculophylla* and *Ulva lactuca*, without highlighting any significant differences in fish growth performance. The assessment of extraction technologies is essential for the sustainable exploitation of bioactive compounds from *Ulva* spp. and other plants. The optimal choice of solvent, time and temperature influences the yield and activity of compounds (Pappou et al., 2022). New extraction technologies, although more efficient and environmentally friendly, present both advantages and challenges to traditional methods, which are mostly manual, require long periods of operation, are consuming toxic and environmentally harmful solvents (Usman et al., 2022). Among the innovative extraction techniques, several methods have gained attention for their efficiency, selectivity, and environmental sustainability.

Enzyme-assisted extraction (EAE) is particularly effective for obtaining proteins and hydrolysates from macroalgae, with the choice of enzyme depending on the desired final product. Although less widely adopted, this technique is recognized for its high extraction yields, compound selectivity, and low energy consumption, while operating under mild and non-toxic conditions (Hardouin et al., 2016; Vasquez et al., 2019).

Ultrasound-Assisted Extraction (UAE) utilizes ultrasound waves with frequencies ranging from 20 kHz to 10 MHz, offering a rapid and straightforward extraction process with low solvent consumption, minimal environmental impact, and high economic efficiency. UAE is particularly suitable for extracting thermolabile bioactive compounds, ensuring their integrity for further processing. This technique provides high yields and excellent efficiency in preserving the bioactivity of extracted compounds (Essa et al., 2018; Rashad et al., 2023).

Supercritical Fluid Extraction (SFE) is an eco-friendly technique known for its high efficiency and rapid extraction rates, using supercritical CO_2 as a solvent. One of the key advantages of SC-CO_2 is its ability to exhibit both gas and liquid properties at the critical point, allowing for effective interaction with target compounds during extraction (Fabrowska et al., 2016). SFE offers mild critical conditions, solvent-free final products, and the selective extraction of desired substances. However, its disadvantages include high equipment costs, complex cleaning procedures, the need for high-pressure operation, and limited solubility of certain fat-soluble compounds, requiring optimization for efficient extraction (Martins et al., 2023).

Microwave-Assisted Extraction (MAE) ensures the rapid and selective extraction of bioactive compounds with low energy and solvent consumption, while also generating minimal waste. This technique is widely applicable and more cost-effective than supercritical fluid extraction, making it a promising method for the natural extracts industry (Mirzadeh et al., 2020; Andre et al., 2023). However, MAE requires additional procedures for the separation of residues of bioactive compounds and can cause damage to their structure due to high temperatures (Martins et al., 2023).

Pressure liquid extraction (PLE) is an advanced, sustainable and efficient development, that provides high yield and fast extraction process (Quitério et al., 2022). This allows the use of various solvents including organic, alkaline, ionic, or hot water liquids, known as hot water pressure extraction (PHWE) or subcritical water extraction (SWE). However, it produces a non-selective extraction of compounds, which can be compensated by the use of adsorbents. Although the initial cost of the equipment is high, it is counterbalanced by the low consumption of solvents and small quantities (Perez-Vazquez et al., 2023).

In conclusion, trends in the development of emerging technologies will continue to grow as sustainable alternatives to address environmental challenges, optimizing extraction methods to reduce energy and resource consumption. Increasing the efficiency of the extraction process can be achieved by combining different technologies, and promoting innovative and promising solutions.

CONCLUSIONS

This evaluation highlighted several insufficiently explored issues that limit the optimal integration of macroalgae as a commercial ingredient in feed for aquaculture animals.

Thus, macroalgal ingredients can partially or totally replace fish meal, with substitution levels ranging from 0% to 100%. The maximum degree of replacement depends on the species of macroalgae used, the feeding habits of fish, and inclusion into diet method.

In conclusion, green macroalgae are a viable option for aquaculture due to the many benefits it offers. In addition to being a sustainable source of nutrients, they contain bioactive compounds that have beneficial effects on the health and growth of farmed fish. The use of green macroalgae in aquaculture feed can help to reduce dependence on conventional ingredients, thereby reducing pressure on wild fish stocks and terrestrial resources. However, further research is therefore needed to assess and optimize their effectiveness, thereby facilitating sustainable application in aquatic production systems.

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FAUNISTIC STUDY ON SOME TERRESTRIAL INVERTEBRATES WITH CONSERVATIVE VALUE FROM COMMUNITY GALBENA AND VEMEŞOAIA, FĂGĂRAŞ MOUNTAINS - ROMANIA

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Abstract

In 2020, a faunistic study on invertebrate fauna from Galbena and Vemeşoaia Community and its adjacent area, from Făgăraş Mountains was accomplished. Galbena and Vemeşoaia Community are spreading on 893 hectares (which represents 0.15% from the Făgăraş Mountains). Even if this property is spread over a small area, five Natura 2000 invertebrates were identified: Rosalia alpina (Linnaeus, 1758); Carabus (Hydrocarabus) variolosus Fabricius, 1787; Pholidoptera transsylvanica (Fischer,1853); Lycaena dispar (Haworth, 1802); Callimorpha (Euplagia) quadripunctata (Poda, 1761), protected under the Habitats Directive 92/43 / EEC. Another endemic species for Romania was identified: Carabus (Megodontus) planicollis Küster, 1827. The numerical abundance of each species was recorded and their areas of distribution were established. In the same time the species threats and pressures were evaluated. The main conservation measures were defined. The present study wants to highlight that even small unstudied area could represents an important conservation point for invertebrate fauna directly and indirectly on their habitats. -

Key words*: conservation, distribution, invertebrates, habitat, pressure, threats.*

INTRODUCTION

In Romanian legislation, community ("obşte") is a form of human organization, whose origin goes back to the beginning of the medieval era. According to this organization, forests and pastures are indivisible property of the entire community. Another characteristic of this form of property is the forbidding of selling to the foreign person, being inherited only by legal successors, from one generation to the next. But this form of property protection is good or not, in terms of Natura 2000 species conservation? The Galbena and Vemeşoaia Community are situated in the South-West part of Făgăraş Mountains, part of ROSCI0122 Făgăraş Mountains. This means that this community is included in European ecological network Natura 2000 (a functional network of protected sites and define appropriate management measures to ensure the restoration or maintenance of species and habitats at a favorable conservation status) (Habitat Directive, 92/43/EEC, 1992; Romanian Official Monitor 2007; 2011). According to the Natura 2000 policy, a selection of some invertebrates' species of community interest was made. In this selection, the following species were included: *Rosalia alpina* (Linnaeus, 1758); *Carabus* (*Hydrocarabus*) *variolosus* Fabricius, 1787; *Pholidoptera transsylvanica* (Fischer, 1853)*; Lycaena dispar* (Haworth, 1802); *Callimorpha* (*Euplagia*) *quadripunctata* (Poda, 1761). We also considered *Carabus* (*Megodontus*) *planicollis* Küster, 1827, an endemic species for Romania (especially Southern Carpathians) (Barloy & Prunar, 2012).

According to management plan of ROSCI0122 Făgăraş Mountains with an area of 198620.5 ha, represents one of the largest sites of community importance at the national level, being located in the central part of Romania, in the administrative area of 4 counties: Sibiu, Braşov, Vâlcea and Argeş. It was designated for the conservation of 27 habitats of interest community (of which 5 are priority) and for 13 invertebrate species (Management Plan of

ROSCI0122 Făgăraș Mountains, 2016). From all invertebrate species with conservative value, four are similar with those signaled in Galbena and Vemeşoaia Community and its adjacent area: *Rosalia alpina, Pholidoptera transsylvanica, Lycaena dispar* and *Callimorpha* (*Euplagia*) *quadripunctata*.

European studies discovered that different management strategies could influence the effectiveness of Natura 2000 network (Rouveyrol & Prima, 2024). Romania shares many conservation concerns with other Eastern and Central European countries. Over 80% of the species of European conservation concern were included in at least one protected area, but plants and invertebrates were underrepresented (Iojă et al., 2010). Cazzolla Gatti et al., 2023 declared that in Romania, the Steppic and the Continental biogeographic regions offer very limited protection to biodiversity and rare species.

If we consider only saproxylic beetles (as: *Rosalia alpina*), the current Natura 2000 network from Romania covers only a small part of their distributions (Mirea et al., 2024). Selective logging and removal of deadwood and old-growth trees, that degraded the forest structure, were frequent negative practices in the Carpathians few decades ago, which lead to a decreasing of abundance of the protected species. In the past, Romanian forest managers oftenly considered them as pest species and applying eradication methods to lower their populations. More actually, these saproxylic beetles are protected, as threatened and endangered species per the EU environmental mandates (Mirea et al., 2024). As a result, forest managers have neither the incentives nor technical information on the saproxylic beetle community to promote concrete conservation actions. Thus, the most important tool in protecting these species was the inclusion of their habitats in the Natura 2000 network (Mirea et al., 2024).

In Romania, the national distributions of those six invertebrates, which constitute the subject of this research were described, from a local level to the national level (Barloy & Prunar, 2012; Iorgu et al., 2015; Lotrean & Manu, 2015; Manu et al., 2014; 2016; 2017; Bărbuceanu, 2017; Iorgu, 2021; Nitzu, 2021, Rákósy & Goia, 2021; Bărbuceanu & Truţă, 2021). In the same time, we identified other studies that revealed their conservation status at national level (Mihăilescu et al., 2015; Iorgu, 2021; Nitzu, 2021; Rákósy, 2021).

The current surface of strictly protected areas from all European countries is unbalanced across biogeographical regions. It does not comply with the 10% target of strict protection, according to the EU 2030 Biodiversity Strategy. In the present only 3% of land is under strict protection. This means that a significant amount of work needs to be done to achieve these conservation goals, considering all geographic and ecological conditions (Cazzolla Gatti et al., 2023).

In this context, any new and original information on the distribution, threats, pressures and conservation measures of invertebrates with conservative value are valuable for protected areas management.

MATERIALS AND METHODS

The study was accomplished in July-August 2020, in Galbena and Vemeşoia Community (GVO), located at South-West of Făgăraș Mountains (Galbena Mountain- N: 45°32'47.5"; E: 024°27'14.0" and Vemeşoaia Mountain- N: 45°32'47.3"; E: 024°26'28.6" E) and its adjacent area (Figure 1). This community is located in Vâlcea County, Romania, on an area of 393 hectares, which represent 0.15% of the Făgăraș Mountains surface. From this area, 328.6 hectares represent the forest ecosystems and the rest are grasslands and riparian zones (Prosilva Geotop SRL., 2015; Manu et al., 2024).

For the inventory of invertebrates' fauna, three methods were used in the field:

- Transect method, using visual searching for adults along transects. Considering the habitat topography, the length of transect was between 100 and 500 m; the width between 10 and 20 m. Transects were carried out in a zigzag pattern to cover all researched areas as best as possible. In the case of small habitats, transects can be shorter and if the species has a very low density, they can be longer. The fauna collection was done by hand or with tweezers, directly on the substrate: soil, rocks, logs, herbaceous vegetation, shrubs and bark of trees. For saproxylic Coleoptera species, in

areas with dead wood (fallen or standing trees, wood stacks), there were investigated the adequate micro-refuges, through removing the bark on the trunks of dry trees and on logs. This method was used for: Orthoptera and Coleoptera species (Iorgu et al., 2015; Lotrean & Manu, 2015; Campanaro et al., 2017).

- Transect method - collection with the entomological net. Transects were established with length by 100 m and the width by 1-1.5 m. This method was used for: Orthoptera, Coleoptera and Lepidoptera species (van Chris et al., 2012; Iorgu et al., 2015; Campanaro et al., 2017).
- Pitfall traps (five traps in each location). Method was used for Coleoptera species (Iorgu et al., 2015; Campanaro et al., 2017).

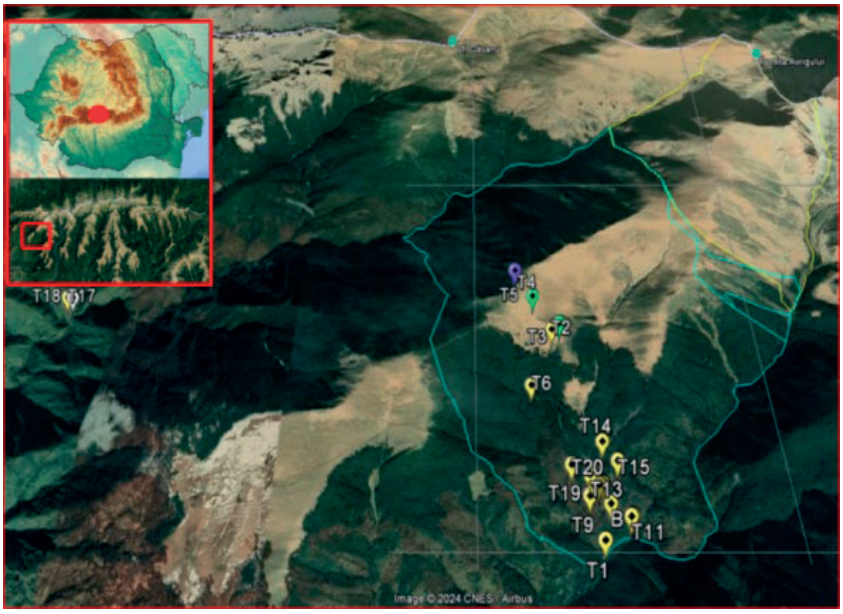


Figure 1. The geographical location of the Galbena and Vemeşoaia Community (blue line), South-West Făgăraş Mountains - Romania (original)

In total twenty two transects were established and two locations with pitfall traps. Transects from Vemeşoaia Mountain recorded an East exposures (Figure 1; Table 1). For taxonomical identification of species, we used an actual key (Panin, 1955; Knechtel & Popovici, 1959; Panin & Săvulescu, 1961; Tolman & Lewington, 2009). Habitats classification was made after Doniţă et al., 2005.

For each protected species, threats and pressures were evaluated (in degree of intensity), using the actual legislation codification: high (for the impacts that have a significantly high negative impact, in the investigated habitat were the species were identified), medium (for the impacts that have a significantly medium negative impact) and low (for the impacts that have a significantly low negative impact) (Habitat Directive, 92/43/EEC, 1992; Romanian Official Monitor, 2018; 2023). In order to differentiate the pressure from threat, we used "the standard lexicon for biodiversity conservation: unified classifications of threats and actions" (Salafsky et al., 2008). According to this lexicon, pressures (stresses) are currently acting and threats are expected in near future. Stresses are attributes of a conservation target's ecology that are impaired directly or indirectly by human activities. Threats can be past (historical), ongoing, and/or likely to occur in the future. Natural phenomena are also regarded as direct threats in some situations.

Table 1. The location of investigated transects and pitfall traps in Galbena and Vemeşoaia Community and its adjacent area from Făgăraş Mountain

Toponym	Habitat Natura 2000	Transect	Altitude
Boia Mare Valley	6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels + 91V0 Dacian Beech forests (*Symphyto-Fagion*)	T1, T11	1033-1066
	6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels	T7, T8	1047-1069
Boia Mică Valley	6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels + 91V0 Dacian Beech forests (*Symphyto-Fagion*)	T17	812
	6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels	T18	798
Vemeşoaia Mountain	4060 Siliceous alpine and boreal grasslands	T2, T3, T4, T5	1772-2007
	9410 Acidophilous Picea forests of the montane to alpine levels (*Vaccinio-Piceetea*)	T6	1502
	91V0 Dacian Beech forests (*Symphyto-Fagion*)	T9, T10, T12	1201-1235
	9410 Acidophilous Picea forests of the montane to alpine levels (*Vaccinio-Piceetea*) - forest edge	T13, T14	1273-1387
	91V0 Dacian Beech forests (*Symphyto-Fagion*) - forest edge	T15, T16, T19, T20	1109-1138
Vemeşoaia Mountain	91V0 Dacian Beech forests (*Symphyto-Fagion*)	Pitfall traps	1226
	9410 Acidophilous Picea forests of the montane to alpine levels (*Vaccinio-Piceetea*)	Pitfall traps	1150
Sterminoasa Valley	91V0 Dacian Beech forests (*Symphyto-Fagion*) + 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels	T21	989
Galbena Mountain	6230* Species-rich Nardus grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe)	T22	1751

RESULTS AND DISCUSSIONS

In Galbena and Vemeşoaia Community (GVO) and on its adjacent area, six species with conservative value were identified (five Natura 2000 species and one endemic). From taxonomical point of view, they were grouped as following: Coleoptera order (*Rosalia alpina, Carabus variolosus, Carabus planicollis*), Lepidoptera order (*Lycaena dispar, Callimoprha quadripunctaria*) and Orthoptera order (*Pholidoptera transsylvanica*) (Figure 2). These species are legaly protected and their conservation status were described at international, national and regional level) (Habitat Directive, 92/43/EEC, 1992; Romanian Official Monitor 2007, 2011; Management Plan of ROSCI0122 Făgăraş Mountains and ROSPA0098 Piemontul Făgăraş, 2016; Murariu & Maican, 2021; IUCN, 2024).

From 22 investigated transects, 13 of them contain favorable Natura 2000 habitats for these invertebrates, as: 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels; 91V0 Dacian Beech forests (*Symphyto-Fagion*); 9410 – Acidophilous Picea forests of the montane to alpine levels (*Vaccinio-Piceetea*); 4060 – Siliceous alpine and boreal grasslands., 6230* – Species-rich Nardus grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe) (Figures 2, 3).

According to Doniţă et al., 2005, habitat 91V0 Dacian Beech forests (*Symphyto-Fagion*) is growing on slopes with medium inclinations and different aspects, plateaus, summits. The bedrock is mainly flish, conglomerates and crystalline schist. The soils are eutricambosol, luvosol from middle-deep to deep and weakly skeletal, moderate poorly acid, humid. The trees layer covers 90-100% and is formed by spruce (*Picea abies*), beech (*Fagus sylvatica*), fir (*Abies alba*), mixed with individuals of sycamore maple (*Acer pseudoplatanus*), elm (*Ulmus glabra*). The shrubs layer is underdeveloped, with rare individuals of *Sambucus racemosa, Daphne mezereum, Rosa* sp. The herbaceous layer varies according with the light touching the ground: *Dentaria*

glandulosa, Galium odoratum, Rubus hirtus, Calamagrostis arundinacea, Luzula luzuloides, etc.

Habitat 9410 – Acidophilous Picea forests of the montane to alpine levels (*Vaccinio-Piceetea*) is growing on very steep slopes with different aspects, peaks, ridges. The bedrock is siliceous and calcareous. The soils are prepodzol, podzol, cryptopodzole, andosol, superficial-middle deep, very acidic, oligobasic, humid. At lower altitude, the trees layer may have 100% coverage, being formed exclusively by spruce (*Picea abies*), or comprising a few individuals of mountain ash (*Sorbus aucuparia*). In the alpine areas, the tree layer covers 60-80%, being formed by 100 years old and 15-20 m height bushes of stone pine (*Pinus mugo*) sau juniper (*Juniperus communis*). The shurbs layer is not present or is underdeveloped: *Sorbus aucuparia, Lonicera nigra, Rubus idaeus*, etc. The herbaceous and undergrowth layer is dominated by *Oxalis acetosella* and *Vaccinium* sp. (Doniţă et al., 2005).

Siliceous alpine and boreal grasslands- 4060 develops on windy areas, slopes and cliffs with N and NE aspects and medium-large inclinations. The bedrock is silica, but also conglomerates with limestones. The soils are superficial, skeletal, lithosol, rankers and cryptopodzoles, humosiosols (under phytocenoses installed secondary), with soil reaction from strong acid to acid (pH = 4.7-5.0). The subshrubs (undergrowth) layer has 20-40 cm height, 80-100% coverage and is dominated by *Rhododendron myrtifolium* (*Rh. kotschy*), *Vaccinium myrtillus, Vaccinium vitis-idaea, Vaccinium gaultherioides*. The herbaceous layer is not distinct. It is intertwined with that of the subshrubs with the following dominant species: *Agrostis capillaris, Festuca rubra, Nardus stricta, Anthoxanthum odoratum, Luzula luzuloides, Potentilla ternata, Homogyne alpina, Loiseleuria procumbens, Geum montanum* (Doniţă et al., 2005).

Habitat 6230* – Species-rich Nardus grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe) is developed on plateaus, slopes, valleys and peaks with gently to moderate slopes, with acidic substrate. Soils are short-profile, spodisoles poor in bases (5-10%), weak aerated and acidic (pH = 3.6-4.5). The trees layer is very scattered, in the grasslands being present sub-shrubs as: *Vaccinium myrtillus, Vaccinium vitis-idaea*. The herbaceous layer is formed by: *Viola declinata, Nardus stricta* (sometimes covering 95% only itself, with a very small number of neighboring species), *Festuca nigrescens*, etc. (Doniţă et al., 2005).

Habitat 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine level is vegetating on steep valleys, shaded gutters and chimneys, sometimes at the edge of the streams. The bedrock is variable. The soil is wet colluvial, rich in fixed gravel and boulder. The arbustive layer is scarce: *Alnus viridis, Salix silesiaca.* The herbaceous layer is dominant, most frequent being *Doronicum austriacum*; there are also present: *Heracleum carpaticum, Heracleum sphondylium, Petasites* sp., *Poa delyii, Phyteuma vagneri, Achillea distans, etc.* (Doniţă et al., 2005).

The Coleoptera species *Rosalia alpina* (Linnaeus, 1758) is protected at national and European level (Romanian Official Monitor, 2007 - annexes III, IVa; Habitat Directive, 92/43/EEC - annexes IIa, IVa). It was declared vulnerable species at international and national level (Nitzu, 2021; IUCN, 2024). According to report under the Article 17 of the Habitats Directive (2013-2018), the overall assesment status of *Rosalia alpina* for Romania, from alpine bioregion is unfavorable-inadequate (European Commison, 2020), as well as at regional level (Management Plan of ROSCI0122 Făgăraş Mountains, 2016). In adjacent area of GVO, this saproxylic species was identified in two locations (T16 and T21), with four individuals (Figures 1, 2). The characteristic habitat of this species is – 91V0 Dacian Beech forests (*Symphyto-Fagion*) - forest edge (Figure 3A). For this species 3 preassures were identified and two threats, with different level of intensity (Table 2).

Species *Carabus variolosus*, Fabricius, 1787 is also protected at national level (Romanian Official Monitor, 2007 - annexes III, IVa) and European level (Habitat Directive, 92/43/EEC - annexes IIa, IVa). In Romania, this species is vulnerable (Nitzu, 2021). The overall assesment of *Carabus variolosus* for Romania,

from alpine bioregion, is favorable (European Commision, 2020). In adjacent area of Galbena and Vemeşoaia Community, this species was identified in a single location, Boia Mică Valley (T18), with one individual (Figures 1, 2). According to Natura 2000, the preffered habitat is 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels (Figure 3B). The presence of forest roads and intensive sheeping grazing are the main pressures for this species. Soil erosion could constitute a threath for this invertebrate (Table 2).

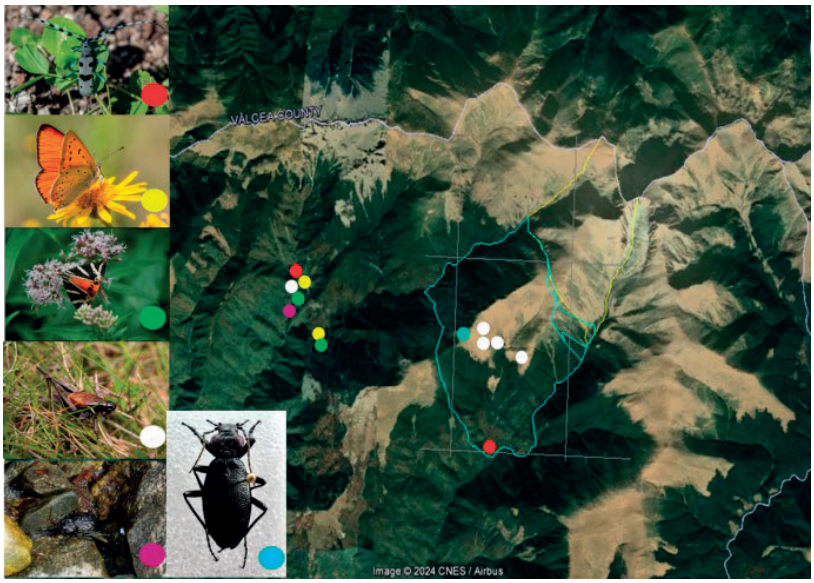


Figure 2. The biogeographically distribution of the Natura 2000 protected invertebrates from Galbena and Vemeşoaia Community and its adjacent area, from Făgăraş Mountain, Romania (red circle = *Rosalia alpina*; purple circle = *Carabus variolosus*; blue circle = *Carabus planicollis*; yellow circle = *Lycaena dispar*; green circle = *Callimoprha quadripunctaria*; white circle = *Pholidoptera transsylvanica*) (original photos: Manu Minodora and Lotrean Nicolae)

Species *Carabus planicollis* Küster, 1827 is an endemic invertebrate for Romania (Southern Carphatians). It lives in the mountain coniferous forests, even in alpine lands (stony alpine meadows), from 1900 to 2000 meters altitude (Barloy & Prunar, 2012; Nitzu, 2021). In Romania, it is endangered species (Nitzu, 2021). In GVO, this species was recorded on Vemeşoaia Mountain (T5), with one individ (Figures 1, 2). The habitat where it was signaled is classify as 4060 – Siliceous alpine and boreal grasslands (Figure 3C). The intensive sheep grazing and soil erosion are the pressure and respectivelly threat for this invertebrate species (Table 2).

In GVO, forest roads are used mainly in summer, in the period when adults of these protected species are active. The presence of access roads causes disturbance to the adjacent habitats, determining a reduction or a fragmenting of the habitat area, pollution and erosion, vegetation destruction, changing of the terrain configuration. These forest roads facilitate the access of people, domestic animals and vehicles. Another pressure observed in the riparian areas 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine level) is the modification of the vegetation structure as a result of the "resining" process (as a method of forest artificial regeneration). This process will determine an increasing of soil acidity and a disappearance of carabids species, which are adapted to the specifical ecological conditions, characteristical for beech forest and for mountain riparian areas. Intensive sheep grazing will disturb flora and fauna, modify the microrelief and will alter the soil quality by compaction.

The butterfly species *Lycaena dispar* (Haworth, 1802) has also an international and national conservation status (Habitat Directive, 92/43/EEC - annexes IIa, IVa; Romanian Official Monitor, 2007 - annexes III, IVa). According to the Article 17 of the Habitats Directive, the overall assesment status of this butterfly species for alpine bioregion from Romania is favorable (European Commison, 2020). This status is in concordance with that given by Rákósy in 2021 at national lavel or that from IUCN (least concern species) (IUCN, 2024; Rákósy, 2021). In Gablena and Vemeşoaia Community, four individuals were identified at Boia Mică Valley (T17) and Sterminoasa Valley (T21) (Figures 1, 2). The characteristic habitat for this species in this small community is a mixture between 6430 Hydrophilous tall herb fringe communities of plains, montane to alpine levels and 91V0 Dacian Beech forests (Symphyto-Fagion) (Figure 3D).

The main pressures for this species are the forest roads and logging without replanting or natural regeneration, which exercise with high and respectively medium intensities. The extensions of these forest roads, construction of forestry ramps and intensive sheep grazing constitute threats for *Lycaena dispar* (Table 2).

Another Natura 2000 protected butterfly species was identified, in the same locations from GVO (T17 and T21): *Callimorpha* (*Euplagia*) *quadripunctata* (Poda 1761), with three individuals (Figure 2). On European level and national it is considered species of community interest whose conservation requires the designation of special areas of conservation (Habitat Directive, 92/43/EEC – annex IIa; Romanian Official Monitor, 2007 - annex III). It conservation status is favorable, for alpine bioregion from Romania, as well as for regional level (Management Plan of ROSCI0122 Făgăraş Mountains, 2016; European Commision, 2020). According to Rákósy, 2021, it is a least concern species. In GVO, the habitats where the species was found were 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels; 91V0 Dacian Beech forests (*Symphyto-Fagion*) (Figure 3E). Forest roads and logging without replanting or natural regeneration are medium intensity pressures for this species. Extensions of these activities represent the main threats (Table 2).

Orthoptera species *Pholidoptera transsylvanica* (Fischer 1853), was identified in five locations from GVO: Vemeşoaia Mountain (T2, T3, T4), Sterminoasa Valley (T21) and Galbena Mountain (T22) (Figures 1, 2). In total, 11 individuals were recorded, in the following habitats: 4060 – Siliceous alpine and boreal grasslands; 91V0 Dacian Beech forests (*Symphyto-Fagion*); 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels; 6230* – Species-rich Nardus grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe) (Figure 3F). It is protected at national and European level (Romanian Official Monitor, 2007- annexes III, IV a; Habitat Directive, 92/43/EEC – annexes IIa, IVa). For alpine bioregion from Romania, this species recorded a favorable conservation status, similar with those obtain at regional level, according to the Management Plan of ROSCI0122 Făgăraş Mountains, in 2016 (European Commision, 2020). Intensive sheep grazing, soil erosion and forest roads are the main pressures for *Pholidoptera transsylvanica* and their extensions, other forestry activities (as construction of forestry ramps), droughts and low rainfall represent the main threats (Table 2).

For the proposed conservation measures, we must consider the pressures and threats listed above for each protected species. Sometimes, these are similar, but they manifest with different intensities (Table 2). For *Rosalia alpina*, we propose that the forest cutting and the wood extraction to be forbidden in the forest area where the species was identified and its neighbor (at least 1 ha) (Campanaro et al., 2017). Forest cuttings will be carried out only in exceptional cases, under strict control, focusing on natural regeneration. Keeping old and dead trees in the forest provides favorable habitat for this species. The removal of dead wood or veteran trees causes a decrease in dead wood availability and negatively affects the survival of *Rosalia alpina*. The access to the forest road will be restricted. Due to the fact that this road is degraded (soil erosion), we propose its stabilization by weeding or by planting beech trees. The construction of new

roads will be avoided (will be accepted when there is no other option and when the new road has a vital importance).

The conservation measures for *Carabus variolosus, Carabus planicollis* and *Pholidoptera transsylvanica* are quite similar, considering the intensive sheep grazing and their transit. This activity must be limited, in the areas where these species were identified. Grazing should be done by rotation, allowing the restoration of the vegetation layer from entire meadow, preventing the appearance and expansion of areas without vegetation, which leads to desertification. We recommend annual sowing of the pasture, with local plant species. In this way the phenomenon of soil erosion will be reduced. Sheeping grazing must be done, considering the support capacity of the meadow, allowing the vegetation recovering (Onete et al., 2021). Pastures from Galbena Mountain recorded a grazing capacity by 2.5 UVM/ha for a period of 85 days/year. Meadows from Vemeşoaia Mountain recorded a grazing capacity by 5 UVM/ha for a period of 70 days/year and those from Sterminoasa Mountain has a grazing capacity of 0.5-2.3 UVM /ha for 85 days/year (Onete et al., 2021).

Table 2: Pressures and threats identified for invertebrates with conservative value, from Galbena and Vemeşoaia Community and its adjacent area, 2020 (pressures and threats codes according to M.O. 304/2018; M.O. 901/2023)

Pressure	**Code**	**Species**	**Pressure intensity for each species**
Roads, paths and railways	D01	*Rosalia alpina* *Carabus variolosus* *Lycaena dispar* *Callimorpha (Euplagia) quadripunctaria* *Pholidoptera transsylvanica*	Medium Medium High Medium Medium
Logging without replanting or natural regeneration	B03	*Rosalia alpina* *Lycaena dispar* *Callimorpha (Euplagia) quadripunctaria*	Medium Medium Medium
Forest cleaning	B 02.02	*Rosalia alpina*	High
Intensive sheep grazing	A 04.01.02	*Carabus variolosus* *Carabus planicollis* *Pholidoptera transsylvanica*	High High High
Soil erosion	K 01.01	*Pholidoptera transsylvanica*	Low
Threat	**Code**	**Species**	**Threat intensity for each species**
Forest road extension	D01	*Rosalia alpina* *Lycaena dispar* *Callimorpha (Euplagia) quadripunctaria* *Pholidoptera transsylvanica*	Medium High High Medium
Logging extension	B03	*Rosalia alpina* *Callimorpha (Euplagia) quadripunctaria* *Pholidoptera transsylvanica*	High Medium Medium
Soil erosion	K01.01	*Carabus variolosus* *Carabus planicollis* *Pholidoptera transsylvanica*	Low Low Low
Other forestry activities (construction of forestry ramps)	BO7	*Lycaena dispar* *Callimorpha (Euplagia) quadripunctaria* *Pholidoptera transsylvanica*	High High Medium
Intensive sheep grazing	A 04.01.02	*Lycaena dispar*	Low
Droughts and low rainfall	M 01.02	*Pholidoptera transsylvanica*	High

In GVO, *Lycaena dispar*, *Callimopha quadripunctaria* and *Pholidoptera transsylvanica* were often identified in riparian areas near to the forest edges or near to forest roads. The conservation of these habitats is essential for species survival and involves limitation of the logging. The forest ramps and the forest exploatation must be placed at one km distance from the habitats of these species. The storage time and the quantity of wood material from forest ramps must be reduced. The lifting of the wooden material should be

done with minimal impact on the adjacent habitats (without soil degradation), with well-maintained equipment. Forest machinery maneuvers (turning, loading, etc.) should be done only on the existing forest roads. The over-dimensioning of forest machinery must be forbidden, so that these maneuvers can be easily carried out in the specially arranged perimeter (the existing forest road), without damaging the neighboring habitats.

A



91V0 Dacian Beech forests (*Symphyto-Fagion*)- forest edge- Vemeşoaia

B



6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels- Boia Mică Valley

C



4060 – Siliceous alpine and boreal grasslands- Vemeşoaia

D



6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels + 91V0 Dacian Beech forests (*Symphyto-Fagion*) – Sterminoasa Valley

E



6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels + 91V0 Dacian Beech forests (*Symphyto-Fagion*) – Boia Mică Valley

F



6230* – Species-rich Nardus grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe)- Galbena

Figure 3. Characteristic Natura 2000 habitats for invertebrate fauna from Galbena and Vemeşoaia Community and its adjacent area (A - *Rosalia alpina*, B - *Carabus variolosus*, C - *Carabus planicollis*, D - *Lycaena dispar*, E - *Callimoprha quadripunctaria*, F- *Pholidoptera trassylvanica*) (original photos: Manu Minodora, Lotrean Nicolae, Nicoară Roxana)

The existing forest roads must be preserved and their extension (in number or dimension) must be forbidden. Roads extension could determine a fragmentation of the characteristically habitats of these invertebrates and soil deterioration. Repairing and maintenance of the forest roads will be carried out with maximum caution, in order to not damage the species' habitats.

For *Lycaena dispar* conservation, the extensive grazing and traditional mowing of the wet areas are two measures for the protection of its habitat. In the period May-October, the grazing will be restricted. The tall hydrophilic grasses and sedges must be protected for the adults mating. This means that the intensive mowing or grazing near to the water courses and in hygrophilous meadows is forbidden.

Schrubs conservation, especially those with *Eupatorium cannabinum* located near to the water courses or in hygrophilous meadows, is another protection method for *Callimoprha quadripunctaria*. Extensive grazing and traditional mowing must be realised, in order to prevent afforestation. These traditional actions will keep the mosaic aspect of the vegetation (areas covered by bushes alternating with areas covered by grassy vegetation). In the same time, the traditional extensive grazing keeps the habitat's heterogenity, alternance between meadows with open areas and shiny rocky places.

Pholidoptera transsylvanica is another species which prefferes riparian areas. Protection of the flowing waters banks, prohibition of waste storage and maintenance of the water quality are essential measures for this species conservation. An adequate grazing management in mountain grasslands, as we mentioned above, will favour the developing of these invertebrates with conservative value.

CONCLUSIONS

In Galbena and Vemeşoaia Community, wich represent 0.15% of the Făgăraş Mountains surface, were identidied six invertebrates with conservative value (five Natura 2000 and one endemic species). If we consider the conservation status at the alpine bioregion of Romania, four invertebrates recorded a favourable conservation status and only one is unfavorable-inadequate. Identification of these protected species demonstrated the presence of favorable, characteristically habitats, as: old, mature deciduous forests with intact forest edges, mountain grasslands, riparian areas with rich hygrophilous vegetation, rivers with flowing water, characterized by a relatively low flow speed and marshy areas in natural forests.

In the same time the five pressures and six threats were evaluated, indicating their intensity. The main preassures were: forest logging, overgrazing, forest roads and soil erosion. The main threats were: the extension of access forest roads, of forest exploitation, builging of new temporaly forest ramps, intensive grazing/overgrazing and desertification. Conservation of these invertebrates implies a decrease in the intensity and/or the disappearance of these pressures and threats, through target protection measures. Application of these conservation measures protects invertebrates on long term as viable biological components of their natural habitats, maintain their distribution areas and provide a favorable characteristically habitats, large enough to keep their populations on long term.

The present study highlighted that even small unstudied area could represents an important conservation hotspot for invertebrate fauna, directly, and indirectly for their habitats.

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BODY INDICES IN COMMON CARP (*Cyprinus carpio*) JUVENILES FED WITH SLUG (*Arion vulgaris*) MEAL AS AN ALTERNATIVE PROTEIN SOURCE

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Abstract

Research of alternative sources of protein for aquaculture feeds is ongoing for many decades, due to the need to make the aquaculture sector more sustainable. This study explores the incorporation of slug (Arion vulgaris) meal as an alternative protein source in the diets of juvenile common carp (Cyprinus carpio) and examines its impact on body indices. Carp juveniles were reared in a controlled environment and fed using a feed containing slug meal as a protein source. The performances were analysed in relation to a control feed that used standard fish meal as an animal protein source. Body indices are analysed in this research such as Fulton condition factor (K), profile index and meatiness indices. The results presented in this article show the potential of slug meal to substitute fish meal in aquaculture feeds. Along with the rising need for sustainable aquaculture practices, investigating unconventional protein sources is crucial for improving fish growth and health while decreasing dependence on traditional fish meal.

Key words*: aquaculture, diet, feed, growth, nutrition.*

INTRODUCTION

Production of food is of central importance for the world population, and aquatic organisms are well represented as food sources. The production of aquatic organisms is represented by capture fisheries and aquaculture. According to the Food and Agriculture Organization, in the year 2022 the production of aquatic organisms from aquaculture surpassed that of capture fisheries, in a historic first (FAO, 2024). This event comes as confirmation of the rising trends in aquaculture production (FAO, 2022). Rising trends in production come together with a careful approach from customers, who are in search of more qualitative products (Lațiu et al., 2024).

In order to make aquaculture more performant, constant improvement of the utilized feeds is paramount, because feed cost is the biggest of the production costs and the market for feed ingredients is experiencing constant price rises (Rana et al., 2009). Of the ingredients utilized in feed production, protein sources represent a large part of the cost. The mainstay in this industry is fish meal (FAO, 2004), due to its highly nutritive nature, but the cost of fish meal is increasingly higher.

Research on alternative sources of protein is split between protein sources of vegetal origin and protein sources of animal origin (Serra et al., 2024). Research on protein sources of animal origin vary from insects (Vrabec et al., 2015), to animal by-products (Luthada-Raswiswi et al., 2021).

Gastropods (class Gastropoda), represent aquatic and terrestrial slugs and snails. This class of invertebrates was researched across decades regarding their nutritional value and potential as animal protein sources in aquaculture (Muntean et al., 2024a).

Among gastropods, slugs seem to be least studied. Tshernyshev et al. (2022) studied slugs for their rearing productivity as a potential food source for human consumption. Meal obtained from slugs as an alternative protein source in aquaculture was investigated by Muntean et al. (2024b), in a study that analyzed the proximate content, polycyclic aromatic hydrocarbons

(PAHs) content and mineral content of slug meal. The Spanish slug, *Arion vulgaris* Moquin-Tandon, 1855 is an invasive species in Romania, and it is considered a pest for the agricultural sector (Zając et al., 2017; Păpureanu et al., 2014; Pfenninger et al., 2014).

The present research comes as a continuum in slug meal research, as a novel alternative protein source for aquaculture productions. In this study, experimental feed containing slug (*Arion vulgaris*) meal was fed to juvenile carp, *Cyprinus carpio* Linnaeus, 1758. In comparison to a control diet containing standard fish meal, body indices such as the Fulton (K) condition factor, profile index (Pi) and meatiness indices (MI1 and MI2) were analyzed.

MATERIALS AND METHODS

This research took place from July 2024 to January 2025. The bioethics commission of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca approved this research with the 401/21.09.2023 decision, issued to serve for the PhD studies of the first author.

For this research, we developed an experimental feed and a control feed. The experimental feed contained slug (*Arion vulgaris*) meal as an animal protein source and the control feed contained fish meal as an animal protein source, which is the standard for aquaculture feeds.

Slugs were gathered during July and August 2024 from the UASMV Cluj-Napoca campus using traps with beer as an attractant. Slug meal was prepared according to the method described by Muntean et al. (2024b). Captured slugs were humanely euthanised using a solution of 50% ethanol and 50% water. Slugs were rinsed with cold water and frozen until further processing. Slug meal was obtained after defrosting the slugs and drying them using a drying oven (UFE 400 oven from Memmert, Büchenbach, Germany) at 60ºC. Mass monitoring using an analytical balance (WSP4000/C/2, Partner Radwag, Radom, Poland) was conducted to determine when the dehydration process was finalized. The dried slugs were then ground to a meal using a mill (PM 100, Retsch GmbH, Haan, Germany). Complementary ingredients to the animal protein sources were chosen to develop the experimental and control feed. Slug meal, fish meal and potential vegetal ingredients to the feed were tested for nutrient content following the modified method of Masithoh et al. (2020), with the Fourier transform near-infrared (FT-NIR) technology using Tango Brucker device (Billerica, MA, USA). After testing various vegetal ingredients, the following were chosen to be used along the animal protein sources: sunflower meal, soybean meal, wheat bran, oat bran, corn germ and tiger nut flour.

Two recipes were developed using the selected vegetal ingredients: one containing slug meal as an animal protein source and the vegetal ingredients; another containing fish meal as an animal protein source and the vegetal ingredients. Proportions of the vegetal ingredients differed, to obtain finite feeds with similar nutrient content and that utilized the same amount of animal protein sources. Each recipe contained 1% vitamin and mineral premix Romzeofort (Romvac, Romania). The experimental feed contained: 30% slug meal, 5% sunflower meal, 20% soybean meal, 5% wheat bran, 5% oat bran, 15% corn germ, 19% tiger nut flour, 1% vitamin-mineral premix. The control feed contained: 30% fish meal, 5% sunflower meal, 10% soybean meal, 10% wheat bran, 15% oat bran, 10% corn germ, 19% tiger nut flour, 1% vitamin-mineral premix. In developing the feeds, we aimed for nutrient contents between the feeds as similar as possible. Pellet feeds with the diameter of 2 mm were obtained using a pelleting machine (Agrofortel AGF-150M, Romania). The experimental and control pellets were then tested in triplicate for their nutritional values using the same method we used for testing the nutritional value of ingredients. The proximate composition of the experimental and control feeds are presented in Table 1.

Table 1. Proximate composition of the control and experimental feeds

Nutrient (%)	Control feed		Experimental feed	
	M ± SEM	SD	M ± SEM	SD
Ash (%)	7.91 ± 2.15	3.04	5.8 ± 0.01	0.01
Carbohydrates (%)	18.10 ± 5.50	7.78	7.37 ± 0.28	0.40
Dry matter (%)	95.82 ± 0.07	0.10	95.02 ± 0.04	0.06
Dietary fiber (%)	28.80 ± 0.17	0.24	32.83 ± 0.09	0.12
Fat (%)	7.53 ± 0.07	0.09	6.8 ± 0.10	0.14
Protein (%)	28.53 ± 0.09	0.13	29.76 ± 0.05	0.07
Total sugars (%)	4.9 ± 0	0.00	3.47 ± 0.03	0.05

Legend: M – mean; SD – standard deviation of the mean; SEM – standard error of the mean.

In preparation to the fish rearing experiment, juvenile carp (*Cyprinus carpio*) acquired from a farm with outdoor ponds from Cluj County were acclimated to laboratory conditions for five weeks. At the start of the experiment, four lots of fish were created with 50 specimens each, with fish being individually sampled based on their weight, to obtain lots that are as uniform as possible. Fish weight ranged from 16.1 grams to 27.9 grams, and the mean weight of the specimens for each lot ranged from 20.43 grams to 20.53 grams. The variance of each lot ranged from 6.52 to 8.78. Thus, we ensured the experiment started with similar lots. The experimental treatments were as follows: treatment A, treatment B and treatment C were fed 2% of their biomass daily using the experimental feed, containing slug meal as an animal protein source. Treatment D was set as the control treatment and was fed 2% of the biomass daily using the control feed, containing fish meal as an animal protein source. All treatments had a day of fasting each week.

The experiment lasted for 40 days and was set up in a controlled environment indoors. Each treatment took place in a 750 liters circular tank, filled with 600 liters of water, that amounted to 65 cm of depth, each fitted with its individual system of filtration. Feeding was conducted twice daily, at the beginning of the light period, and before the end of the light period, which lasted for 8 hours daily. Temperature ranged between 19.3ºC and 21.3ºC, and pH ranged between 7.18 and 7.37. Temperature and pH were sampled daily at the beginning of the light period. Dissolved oxygen was sampled weekly and ranged between 7.96 mg O_2/l and 9.08 mg O_2/l. After 23 days an intermediary measurement of all specimens was conducted, scheduled for the nearest fasting day near the middle of the experiment. At the end of the experiment, the final measurement was conducted. The initial measurement at the beginning of the experiment, the intermediary measurement and the final measurement were conducted in the same manner: each fish from each treatment was individually weighed (0.1 grams precision) and photographed in a specially designed fish tank that allows to photograph the fish against the aquarium glass which is fitted with a millimetric scale and is always at the same distance from the camera. Based on the photographs, somatic measurements were determined using the AmScope ToupView 3.7 software. The method of Lațiu et al. (2022) was utilized and the following measurements were determined: total length (cm), standard length (cm), commercial length (cm), fork length (cm), maximum height (cm), minimum height (cm), head length (cm), caudal peduncle length (cm).

Using the weight of the specimens and the somatic measurements, we proceeded to calculating the body indices of the specimens for the purpose of this study.

The Fulton's (K) condition factor was determined using Froese (2006) formula:

$$K = 100 * \frac{W}{Tl^3}$$

where: K is Fulton's condition factor; W = body weight (g); Tl = total length (cm).

The profile index (PI) was calculated based on Cocan and Mireșan (2016) formula:

$$PI = \frac{Sl}{H}$$

where: PI is profile index; Sl = standard length (cm); H = maximum height (cm).

The first meatiness index (MI1) was calculated using the formula from Cocan et al. (2024):

$$MI1 = \frac{Hl * 100}{Sl}$$

where: MI1 is meatiness index 1; Hl = head length (cm); Sl = standard length (cm).

The second meatiness index (MI2) was calculated using the formula from Cocan et al. (2024):

$$MI2 = \frac{Pl * 100}{Sl}$$

where: MI2 is meatiness index 2; Pl = caudal peduncle length (cm); Sl = standard length (cm).

During the experiment, no fatalities were recorded for each treatment.

Data was analysed using Microsoft Excel and GraphPad Prism 8.0.2. The analysis for the indices evolution was determined between the treatments inside the measurement phases, as well as individually for each treatment across the measurement phases. Before testing the data, data were checked for normal distribution using the Shapiro-Wilk test, along with the descriptive statistics (minimum, maximum, mean ±

standard deviation, median, standard error of mean). Data were analysed in conformity with their distribution (non-parametric), using the Kruskal-Wallis test. Where the test showed statistically significant differences, the Dunn post-hoc test for multiple comparisons was utilized, with the purpose of precisely identifying the data that cause the statistically significant differences. The significance threshold was $\alpha = 0.05$.

The indices were calculated for the initial population, which consisted of all the initial lots analysed together (200 specimens), and for each treatment during each phase (measurement): initial, intermediary and final.

Results regarding the body indices are presented graphically and the following notations were utilized in various combinations in text and graphs: K – Fulton's condition factor; PI – profile index; MI1 – meatiness index 1; MI2 – meatiness index 2; pop. start – initial population; A – experimental treatment A; B – experimental treatment B; C – experimental treatment C; D – control treatment D; initial – initial measurement; intermediary – intermediary measurement; final – final measurement.

RESULTS AND DISCUSSIONS

The descriptive statistics for each treatment during each phase (measurement) and for the initial population are presented in Table 2.

For the Fulton's (K) condition factor, no statistically significant differences were found when comparing between treatments inside each phase (Figure 1).

When analysing the evolution of the Fulton's (K) condition factor for the same treatment across all measurement phases, the following statistically significant differences were encountered between initial measurement and intermediary measurement and between initial measurement and final measurement: $K_{A\ initial}$ vs. $K_{A\ intermediary}$ (***, p=0.0001); $K_{A\ initial}$ vs. $K_{A\ final}$ (****, p<0.0001); $K_{B\ initial}$ vs. $K_{B\ intermediary}$ (****, p<0.0001); $K_{B\ initial}$ vs. $K_{B\ final}$ (****, p<0.0001); $K_{C\ initial}$ vs. $K_{C\ intermediary}$ (****, p<0.0001); $K_{C\ initial}$ vs. $K_{C\ final}$ (****, p<0.0001); $K_{D\ initial}$ vs. $K_{D\ intermediary}$ (****, p<0.0001); $K_{D\ initial}$ vs. $K_{D\ final}$ (****, p<0.0001). The drop in values of the condition factor can be attributed to antinutritional factors (i.e. feed structure) or environmental conditions (i.e. water quality), that occurred in the accommodation of the carp juveniles to the experimental conditions, although they were acclimated for five weeks to laboratory conditions from outdoor pond conditions prior to the experiment. No statistically significant differences were found when analysing the evolution of the Fulton's (K) condition factor for the same treatment between the intermediary and the final measurement.

Hwang et al. (2016) obtained during their research condition factor values between 1.93±0.20 and 2.48±0.37 for juvenile carp (*Cyprinus carpio*) that were reared from hatching in a controlled environment at 23-25°C. A mean value of Fulton's condition factor of 1.82±0.011 for adult wild common carps was obtained by Cocan et al. (2024) and mean values for three populations of adult common carps between 2.04±0.18 and 3.81±0.39 were obtained by Savin et al. (2022).

Lower mean values were recorded in this study, between 1.4 ± 0.16 and 1.88 ± 0.22, compared to the literature.

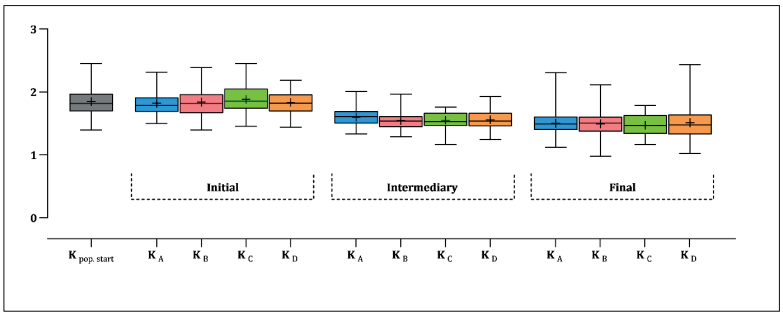


Figure 1. Fulton's (K) condition factor during each phase of the experiment and for the initial population

Table 2. Descriptive statistics for the initial population and each treatment during each phase

	$K_{pop.\ start}$	$K_{A\ initial}$	$K_{B\ initial}$	$K_{C\ initial}$	$K_{D\ initial}$	$K_{A\ intermediary}$	$K_{B\ intermediary}$	$K_{C\ intermediary}$	$K_{D\ intermediary}$	$K_{A\ final}$	$K_{B\ final}$	$K_{C\ final}$	$K_{D\ final}$
N	200	50	50	50	50	50	50	50	50	50	50	50	50
Minimum	1.40	1.50	1.40	1.46	1.44	1.33	1.29	1.17	1.24	1.12	0.98	1.16	1.02
Maximum	2.45	2.32	2.39	2.45	2.18	2.01	1.97	1.76	1.93	2.31	2.11	1.79	2.43
M ± SEM	1.84±0.01	1.82±0.03	1.83±0.03	1.88±0.03	1.83±0.03	1.59±0.02	1.55±0.02	1.55±0.02	1.56±0.02	1.50±0.03	1.4±0.02	1.47±0.02	1.5±0.04
Median	1.82	1.79	1.82	1.85	1.82	1.61	1.54	1.53	1.54	1.49	1.51	1.47	1.48
SD	0.20	0.02	0.22	0.22	0.18	0.14	0.13	0.13	0.14	0.17	0.16	0.16	0.30
	$PI_{pop.\ start}$	$PI_{A\ initial}$	$PI_{B\ initial}$	$PI_{C\ initial}$	$PI_{D\ initial}$	$PI_{A\ intermediary}$	$PI_{B\ intermediary}$	$PI_{C\ intermediary}$	$PI_{D\ intermediary}$	$PI_{A\ final}$	$PI_{B\ final}$	$PI_{C\ final}$	$PI_{D\ final}$
N	200	50	50	50	50	50	50	50	50	50	50	50	50
Minimum	2.25	2.42	2.25	2.41	2.40	2.38	2.31	2.44	2.47	2.44	2.34	2.42	2.48
Maximum	3.33	3.00	3.33	2.90	3.04	3.14	3.10	2.99	3.00	3.17	3.34	3.07	2.90
M ± SEM	2.68±0.01	2.68±0.02	2.70±0.02	2.66±0.02	2.69±0.02	2.71±0.02	2.72±0.02	2.70±0.02	2.70±0.02	2.72±0.02	2.73±0.02	2.72±0.02	2.67±0.01
Median	2.70	2.70	2.75	2.67	2.69	2.72	2.74	2.71	2.68	2.74	2.70	2.74	2.66
SD	0.14	0.12	0.17	0.12	0.14	0.13	0.13	0.12	0.11	0.16	0.17	0.15	0.09
	$MI1_{pop.\ start}$	$MI1_{A\ initial}$	$MI1_{B\ initial}$	$MI1_{C\ initial}$	$MI1_{D\ initial}$	$MI1_{A\ intermediary}$	$MI1_{B\ intermediary}$	$MI1_{C\ intermediary}$	$MI1_{D\ intermediary}$	$MI1_{A\ final}$	$MI1_{B\ final}$	$MI1_{C\ final}$	$MI1_{D\ final}$
N	200	50	50	50	50	50	50	50	50	50	50	50	50
Minimum	26.12	26.26	26.12	26.55	27.38	28.23	28.57	28.08	31.00	28.02	29.17	29.79	29.55
Maximum	40.25	39.24	37.64	40.25	36.81	39.04	36.66	38.88	37.72	37.44	38.34	39.29	37.12
M ± SEM	32.57±0.2	33.04±0.42	32.61±0.39	32.29±0.4	32.35±0.36	33.02±0.35	33.25±0.28	33.28±0.34	34.38±0.21	33.41±0.26	33.66±0.29	34.81±0.27	33.65±0.28
Median	32.94	33.32	32.99	32.43	32.93	33.08	33.30	33.71	34.39	33.85	33.90	35.09	33.99
SD	2.77	2.95	2.75	2.82	2.54	2.47	2.01	2.37	1.47	1.82	2.02	1.91	1.95
	$MI2_{pop.\ start}$	$MI2_{A\ initial}$	$MI2_{B\ initial}$	$MI2_{C\ initial}$	$MI2_{D\ initial}$	$MI2_{A\ intermediary}$	$MI2_{B\ intermediary}$	$MI2_{C\ intermediary}$	$MI2_{D\ intermediary}$	$MI2_{A\ final}$	$MI2_{B\ final}$	$MI2_{C\ final}$	$MI2_{D\ final}$
N	200	50	50	50	50	50	50	50	50	50	50	50	50
Minimum	15.31	15.86	15.31	17.21	16.28	17.18	16.34	16.21	15.88	17.89	16.45	16.34	14.74
Maximum	22.87	22.87	22.67	20.72	22.64	21.86	20.67	20.85	19.43	22.25	21.44	21.15	20.82
M±SEM	18.95+0.09	18.84+0.19	18.87+0.18	19.02+0.13	19.08+0.21	19.25+0.17	18.57+0.14	18.52+0.15	17.81+0.15	19.83+0.14	18.86+0.17	17.83+0.14	18.36+0.17
Median	18.97	18.77	18.85	19.00	19.04	19.34	18.64	18.55	17.89	19.81	18.83	17.89	18.36
SD	1.26	1.36	1.30	0.90	1.45	1.18	0.95	1.09	1.04	0.97	1.21	1.00	1.21

Legend: N – sample size; M – mean value; SD – standard deviation of the mean; SEM – standard error of the mean; pop. start – initial population; initial – refers to the initial measurement; intermediary – refers to intermediary measurement; final – refers to final measurement; K – Fulton's condition factor; PI – profile index; MI1 – meatiness index 1; MI2 – meatiness index 2.

The profile index (PI) analysis showed no statistically significant differences when comparing between treatments inside each phase (Figure 2).
Statistically significant differences were not encountered for the profile index (PI) when analysing the evolution of the same treatment across the initial, intermediary and final measurement phases.

Lower values of the profile index (PI) show a greater amount of meat on the carcass. Cocan et al. (2024) obtained a mean value of 2.4 ± 0.013 for the profile index of adult wild common carp, which is a close value to the mean values obtained in this study for juvenile carp, between 2.66 ± 0.12 and 2.73 ± 0.17, considering the age difference from the studies.

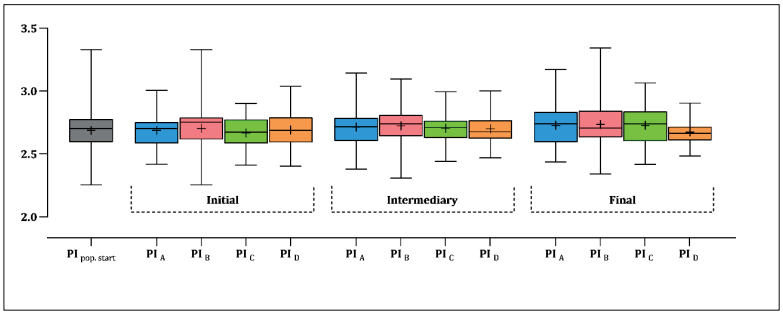


Figure 2. Profile index (PI) during each phase of the experiment and for the initial population

The analysis of the meatiness index 1 (MI1) showed no statistically significant differences when comparing the treatments inside each phase (Figure 3).
When comparing the evolution of the same treatment for the meatiness index 1 (MI1), statistically significant differences were encountered for treatment C, between $MI1_{C\ initial}$ vs. $MI1_{C\ final}$ (****, $p<0.0001$) and $MI1_{C\ intermediary}$ vs. $MI1_{C\ final}$ (*, $p = 0.0466$), and for treatment D, between the initial and intermediary measurement, $MI1_{D\ initial}$ vs. $MI1_{D\ intermediary}$ (**, $p=0.004$). The value of the meatiness index 1 (MI1) did not improve for the mentioned treatments.
The results from this study regarding the meatiness index 1 (MI1) show mean values between 32.29 ± 2.82 and 34.81 ± 1.91, which is a higher value than the mean obtained by Cocan et al. (2024) for adult wild common carp. A higher value of the meatiness index 1 translates to a lower amount of meat on the carcass.

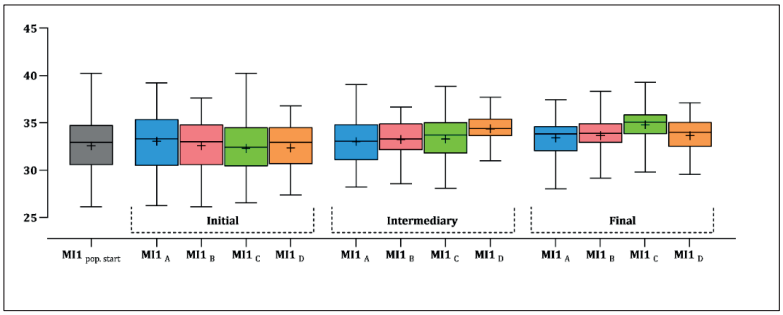


Figure 3. Meatiness index 1 (MI1) during each phase of the experiment and for the initial population

The analysis of the meatiness index 2 (MI2) showed statistically significant differences for the intermediary measurement between $MI2_{A\ intermediary}$ vs. $MI2_{D\ intermediary}$ (****, $p<0.0001$) (Figure 4). For the final measurement statistically significant differences were found

between $MI2_{A\ final}$ vs. $MI2_{B\ final}$ (**, p=0.0047), between $MI2_{A\ final}$ vs. $MI2_{C\ final}$ (****, p<0.0001), between $MI2_{A\ final}$ vs. $MI2_{D\ final}$ (****, p<0.0001) and between $MI2_{B\ final}$ vs. $MI2_{C\ final}$ (**, p = 0.0014). These differences can be attributed to the fact that the measurement of the caudal peduncle is the most sensible to human error, because it is subjective to establish which is the anterior point of the measurement.

The evolution of the same treatment across measurement phases shows statistically significant differences for treatment A, between $MI2_{A\ initial}$ vs. $MI2_{A\ final}$ (**, p = 0.0022), for treatment C, between $MI2_{C\ initial}$ vs. $MI2_{C\ final}$ (****, p<0.0001), and for treatment D, between $MI2_{D\ initial}$ vs. $MI2_{D\ intermediary}$ (***, p = 0.0003).

In the case of treatment A, the value of meatiness index 2 (MI2) did not improve. For treatment C there was a significant improvement in value between the initial measurement and the final measurement, and for treatment D there was a significant improvement in value between the initial measurement and the intermediary measurement.

Mean values of the meatiness index 2 (MI2) from this research are between 17.81 ± 1.04 and 19.83 ± 0.97, which is a slightly lower value than that obtained by Cocan et al. (2024) of 20.04 ± 0.16 for adult wild common carp. The lower the value of the meatiness index 2 (MI2) is, the bigger is the meat content on the carcass.

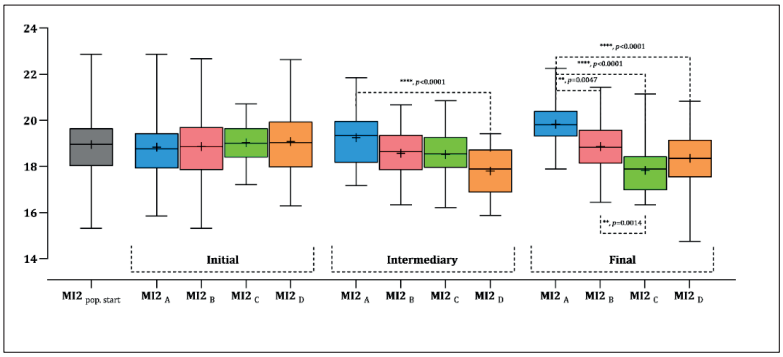


Figure 4. Meatiness index 2 (MI2) during each phase of the experiment and for the initial population (**, p<0.005; ****, p<0.0001)

To our knowledge, this is the first experimental application of slug meal in fish feeding trials assessing morphometric performance. This investigation broadens the scope of alternative proteins in aquaculture and highlights the unexplored potential of non-insect invertebrates in feed innovation.

The use of terrestrial gastropods collected from agricultural environments could serve as a biological control measure while simultaneously contributing to protein circularity. This dual role adds value to what is traditionally considered a pest, reinforcing the principles of resource efficiency and waste minimization.

CONCLUSIONS

The analysis of body indices can determine how different approaches to diet can influence the growth of aquatic organisms. Based on the results of this research on body indices of common carp (*Cyprinus carpio*) juveniles reared in a controlled environment using an experimental feed containing slug meal (*Arion vulgaris*) in comparison to standard fish meal, as an animal protein source, it can be concluded that slug meal can replace fish meal in feed with similar results.

The research demonstrates the feasibility of integrating unconventional protein sources, such as slug meal, into aquaculture feed without compromising fish condition. Our findings support future investigations into local, low-cost protein alternatives for sustainable aquaculture.

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LAVENDER, A NATURAL ADDITION TO FISH DIET, ENHANCEMENTS IN GROWTH AND IMMUNE SYSTEM

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Abstract

Lavender, widely recognized for its medicinal properties, has gained attention for its potential to enhance fish health in aquaculture. This paper resumes current knowledge on the effects of lavender extracts, highlighting their antioxidant properties and immune-enhancing capabilities on fish. Evidence from recent studies indicates that lavender extracts efficiently reduce oxidative stress and improve immune responses in fish. These findings suggest that incorporating lavender extracts into aquaculture practices could reduce reliance on synthetic additives and antibiotics, supporting more sustainable and environmentally friendly approaches. Overall, lavender emerges as a promising natural alternative for promoting fish health and promoting eco- friendly aquaculture practices.

Key words*: antioxidant effects, essential oil, fish health, lavender extracts, medicinal plants.*

INTRODUCTION

Aquaculture is one of the fastest-growing sectors within global food production, driven largely by the rising demand for aquatic products. This rapid expansion has brought about significant environmental and health-related challenges due to extensive reliance on synthetic chemicals such as antibiotics and pharmaceuticals, primarily used for disease control and enhancing fish health (Boaru et al., 2022; FAO, 2022). However, the detrimental ecological impacts, including antibiotic resistance, accumulation of toxic residues in animal tissues, and disruptions to ecological balance, have heightened public awareness and increased scrutiny from regulatory bodies, accelerating the search for sustainable, natural alternatives (Filep et al., 2016; Boaru et al., 2022; De la Cruz et al., 2020).

Intensification of aquaculture practices demands optimal water quality management and robust fish health maintenance. Synthetic chemical agents frequently utilized for disease prevention, stress mitigation, and growth improvement, particularly during transport or juvenile stages, pose significant ecological risks through environmental persistence and bioaccumulation (De la Cruz et al., 2018). Despite initial efficacy, these synthetic chemicals often result in oxidative stress, tissue damage, and pathological lesions in aquatic species, negatively affecting productivity and incurring substantial economic losses (De la Cruz et al., 2020; Morteza et al., 2020). Consequently, adopting eco-friendly alternatives is essential for sustainable aquaculture management.

Currently, there is increasing consumer demand globally, especially in markets such as the United States, Asia, and the European Union, for natural and organic aquaculture products. This trend is driven by heightened consumer preferences for healthier food options produced in environmentally sustainable ways (Ndakalimwe, 2019). Medicinal plants offer significant promise as alternative solutions due to their wide-ranging beneficial effects, including improved growth performance, enhanced immune responses, antimicrobial activities, and better overall health in farmed aquatic species (Antache et al., 2014; Farag et al., 2021).

Plants belonging to the Lamiaceae family have gained considerable attention within aquaculture research due to their diverse biological properties. These include antioxidant, anti-inflammatory, immunostimulant, antiviral, antiparasitic, and antihelminthic effects, which collectively

contribute to enhanced fish health, improved disease resistance, and greater feed conversion efficiency (Bahaciu et al., 2018; Antache et al., 2014; Farag et al., 2021). Utilization of these plant-based solutions aligns closely with environmental conservation and public health concerns, providing aquaculture with sustainable management strategies.

Among medicinal plants suitable for aquaculture applications, lavender (*Lavandula angustifolia* Mill.) stands out due to its rich content of bioactive compounds and extensive historical use in traditional medicine (Muntean, 2016). Lavender is indigenous to Mediterranean regions and North Africa and has been recognized and utilized therapeutically since ancient times across various civilizations (Muntean, 2016). Despite its historical medicinal significance, its application in aquaculture is relatively novel but promising.

Lavender extracts exhibit strong antimicrobial properties, effectively inhibiting pathogenic bacteria and other microorganisms commonly encountered in aquaculture systems (Wimalasena et al., 2018). The antimicrobial activity of lavender essential oils primarily derives from constituents such as linalool and linalyl acetate, which effectively combat a broad spectrum of pathogens, including bacteria and parasites significant to aquaculture health management (Wimalasena et al., 2018; Perez et al., 2017; Bahmani et al., 2014; Bahaciu et al., 2021).

In addition to antimicrobial effects, lavender has demonstrated antihelminthic capabilities, significantly aiding in controlling parasitic infestations prevalent in aquaculture. Parasitic infections negatively impact fish growth, survival rates, and overall productivity, thus representing a critical management challenge in fish farming operations (Perez et al., 2017; Bahmani et al., 2014). Utilizing lavender-based treatments could substantially reduce reliance on chemical antiparasitic agents, thereby minimizing ecological risks and avoiding residual chemical accumulation in aquatic organisms (Bahmani et al., 2014).

Lavender's immunomodulatory effects further amplify its potential in aquaculture by enhancing fish immune responses and resistance to diseases and stressors. The plant's extracts mitigate inflammation and oxidative stress, strengthening physiological resilience and reducing vulnerability to environmental and physiological stressors, crucial during fish transport, handling, or exposure to suboptimal water quality (Saeed et al., 2022; Fathem et al., 2020).

The anti-inflammatory properties of lavender contribute significantly to managing stress-induced health conditions commonly experienced in intensive aquaculture systems. Stress mitigation through lavender supplementation aids in maintaining physiological balance, improving overall fish welfare and productivity, and reducing economic losses associated with stress-induced disease outbreaks (Saeed et al., 2022).

Therefore, incorporating lavender extracts as dietary supplements in aquaculture represents a promising strategy for sustainable fish farming practices. Lavender's integration as a phytoadditive can considerably reduce dependency on synthetic antibiotics and chemicals, promoting safer and more environmentally friendly aquaculture operations. Additionally, the adoption of lavender aligns well with global initiatives aimed at promoting sustainable and responsible food production, potentially enhancing consumer acceptance, reducing environmental impacts, and improving animal welfare standards.

This study aims to synthesize existing scientific data concerning lavender's potential applications in aquaculture, specifically focusing on its antimicrobial and immunomodulatory properties. By examining key areas such as disease prevention, immune enhancement, and improved growth performance, this review underlines practical applications and benefits of lavender supplementation in aquaculture feeds, ultimately contributing to sustainable, eco-friendly, and economically viable aquaculture systems.

MATERIALS AND METHODS

Online databases such as Web of Science, Google Scholar, Researchgate and ScienceDirect, were used to collect published data regarding medicinal plants especially

lavender. Its effects, in the context of aquaculture and fish farming was prior. The reference list of each retrieved article was examined for other relevant studies related to this topic.

Articles published in english were selected, focusing on studies involving the use of lavender as a feed additive, but also including its application as an anaesthetic, water additive, or for other uses in aquaculture.

Both research articles and review papers were included in this study.

RESULTS AND DISCUSSIONS

PHYTOCHEMICAL COMPOSITION AND BIOACTIVE PROPERTIES OF LAVENDER

Lavender is rich in bioactive compounds that contribute to its medicinal and therapeutic properties. Its essential oil (LEO) comprises a diverse range of phytochemicals, with linalool, linalyl acetate, 1,8-cineole (cineole), camphor, borneol, and rosmarinic acid being the predominant constituents, each exhibiting significant biological activity (Habán et al., 2023; Prusinowska et al., 2014; Lis-Balchin, 2002). Among these, linalool and linalyl acetate stand out due to their potent antimicrobial, anxiolytic, and immunomodulatory effects, making lavender an attractive natural additive for aquafeeds (Alam et al., 2024; Yousefi et al., 2020).

Gas chromatography-mass spectrometry (GC-MS) analysis has consistently identified linalool as the most abundant compound in lavender essential oil, comprising approximately 25-45% of the total oil content, followed by linalyl acetate at 25-30% (Abdel-Rahim et al., 2024). These compounds enhance fish immunity, promoting growth, and mitigating oxidative stress (Boaru et al., 2022; Peana et al., 2002). Other bioactive components, such as geraniol, have demonstrated antimicrobial and anti-inflammatory properties, further supporting fish health and resilience against pathogens (Paray et al., 2020).

Lavender's phytochemical profile consists of a complex blend of essential oils, polyphenols, and flavonoids, contributing to its antimicrobial, anti-inflammatory, and immunostimulatory effects (Prusinowska et al., 2014; Habán et al., 2023). The primary bioactive compounds identified through GC-MS and their respective biological roles are summarized in the table below (Table 1).

Table 1. Primary bioactive compounds of lavender and their biological roles

Compound	Percentage in Essential Oil	Key Biological Effects	References
Linalool	25-45%	Antimicrobial, antioxidant, immunomodulatory	Abdel-Rahim et al., 2024; Boaru et al., 2022; Peana et al., 2002
Linalyl acetate	25-30%	Stress-reducing, anti-inflammatory	Abdel-Rahim et al., 2024; Yousefi et al., 2020
Cineole (1,8-cineole)	5-10%	Respiratory health, immune-enhancing	Habán et al., 2023
Camphor	2-7%	Antibacterial, antifungal, analgesic	Prusinowska et al., 2014; Habán et al., 2023
Borneol	1-5%	Wound healing, sedative properties	Prusinowska et al., 2014; Habán et al., 2023
Rosmarinic acid	1-4%	Antioxidant, anti-inflammatory, antiviral	Paray et al., 2020

Linalool and linalyl acetate, the two dominant constituents, are particularly responsible for lavender's antimicrobial efficacy and stress-mitigating properties(Abdel-Rahim et al., 2024). These compounds enhance immune function by modulating cytokine activity and improving phagocytic responses in fish, making lavender a promising phytoadditive in aquaculture (Boaru et al., 2022; Peana et al., 2002).

Antioxidant properties of lavender

Lavender essential oil (LEO) is well-documented for its potent antioxidant properties, which play a crucial role in mitigating oxidative stress in aquatic organisms (Habán et al., 2023; Hajirezaee et al., 2022). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the efficiency of the antioxidant defense system in neutralizing these harmful molecules (Hoseini & Yousefi, 2019). ROS, including superoxide anions, hydrogen peroxide, and hydroxyl radicals, can cause severe cellular damage by oxidizing proteins,

lipids, and DNA, leading to compromised physiological functions, immune suppression, and reduced growth performance in fish (Yousefi et al., 2019; Abdel-Rahim et al., 2024).

Numerous studies have demonstrated that lavender possesses strong free radical scavenging activity, which contributes to its ability to protect fish against oxidative damage (Yousefi et al., 2020). The bioactive compounds in lavender, particularly linalool, linalyl acetate, and rosmarinic acid, exhibit significant antioxidant potential by neutralizing ROS and enhancing the endogenous antioxidant defense system in aquatic species (Paray et al., 2020; Hajirezaee et al., 2022). These phytochemicals have been shown to upregulate the expression and activity of key enzymatic antioxidants, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), which play essential roles in detoxifying ROS and maintaining cellular homeostasis (Hoseini & Yousefi, 2019; Yousefi et al., 2019).

Dietary inclusion of lavender in fish feed has been linked to increased levels of CAT, SOD, and GPx, indicating enhanced oxidative defense mechanisms (Abdel-Rahim et al., 2024). These enzymes work synergistically to decompose ROS; SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide, which is then broken down by CAT and GPx into water and oxygen, thereby preventing oxidative damage to cellular components (Yousefi et al., 2019). Additionally, fish supplemented with lavender-based diets exhibit a significant reduction in malondialdehyde (MDA) levels, a widely recognized biomarker of lipid peroxidation, confirming its protective role against oxidative stress-induced cellular deterioration (Paray et al., 2020).

The antioxidative effects of lavender are particularly beneficial in aquaculture, where environmental stressors such as high stocking densities, poor water quality, and transport-induced stress can elevate ROS production and increase disease susceptibility (Hajirezaee et al., 2022; Yousefi et al., 2020). By reducing oxidative stress, lavender supplementation not only improves fish health and immune function but also enhances overall growth performance and feed efficiency, supporting the sustainability of aquaculture practices (Hoseini & Yousefi, 2019; Boaru et al., 2022).

Immunomodulatory effects of lavender

Lavender (*Lavandula angustifolia*) has gained attention in aquaculture for its immunostimulatory properties, primarily attributed to its bioactive compounds, including linalool, linalyl acetate, and 1,8-cineole (Hajirezaee et al., 2022; Hoseini & Yousefi, 2019). These compounds have been shown to enhance both innate and adaptive immune responses in fish, thereby improving disease resistance and overall health (Abdel-Rahim et al., 2024).

Dietary supplementation with lavender essential oil (LEO) has been found to significantly elevate key immune parameters in various fish species. For example, in European seabass (*Dicentrarchus labrax*), fish fed lavender-enriched diets exhibited increased levels of total immunoglobulin (IgM), respiratory burst activity (RBA), lysozyme activity, and complement component C3, all of which are critical in pathogen defense and immune system regulation (Abdel-Rahim et al., 2024; Ahmadi et al., 2014). Lysozyme is an essential enzyme involved in the innate immune response, directly targeting bacterial cell walls and enhancing phagocytosis, while complement proteins, such as C3, contribute to immune signaling and the destruction of pathogens (Banaee et al., 2011; Hoseini & Yousefi, 2019).

Lavender supplementation has been shown to modulate immune-related gene expression in fish. Studies have demonstrated that fish receiving lavender-based diets exhibit upregulated expression of inducible nitric oxide synthase (iNOS) and complement factor C3, both of which play key roles in immune defense mechanisms (Hajirezaee et al., 2022). iNOS is involved in the production of nitric oxide (NO), a crucial molecule in immune responses that enhances macrophage activity and antimicrobial defense (Paray et al., 2020). The upregulation of C3 further supports fish immunity by promoting the activation of the complement system, a fundamental component of innate immune protection (Ahmadi et al., 2014).

The immunostimulatory effects of lavender are likely due to the synergistic actions of its active compounds, particularly linalool and cineole, which have been shown to enhance immune cell function and reduce inflammation in aquatic organisms (Hoseini & Yousefi, 2019; Abdel-Rahim et al., 2024). Linalool is known for its anti-inflammatory and immunomodulatory properties, contributing to reduced oxidative stress and enhanced leukocyte activity (Dawood et al., 2022). Meanwhile, cineole has been reported to increase cytokine production and improve fish resistance to bacterial and parasitic infections (Yousefi et al., 2020).

Lavender supplementation has been found to reduce stress, a common challenge in aquaculture, particularly in intensive farming conditions where fish are exposed to high stocking densities, handling stress, and environmental fluctuations (Hajirezaee et al., 2022; Yousefi et al., 2019). Chronic stress suppresses immune function by elevating cortisol levels, which negatively impact leukocyte activity and reduce disease resistance in fish (Banaee et al., 2011).

Lavender-based phytoadditives in aquaculture diets represents a promising strategy for enhancing fish health while reducing reliance on synthetic immunostimulants and antibiotics (Abdel-Rahim et al., 2024; Ahmadi et al., 2014). By modulating innate immune responses and gene expression, lavender offers a natural and effective approach to strengthening fish immunity, minimizing disease outbreaks, and promoting sustainable aquaculture practices (Hajirezaee et al., 2022; Hoseini & Yousefi, 2019).

Antimicrobial and antiparasitic activity

Lavender essential oil (LEO) possesses potent antimicrobial and antiparasitic properties, making it a promising natural alternative to conventional antibiotics and chemical treatments in aquaculture (Wimalasena et al., 2018; Hoseini & Yousefi, 2019). The bioactive compounds in lavender, particularly linalool, linalyl acetate, and 1,8-cineole, have been shown to exert strong antimicrobial effects against a range of Gram-positive and Gram-negative bacterial pathogens commonly affecting fish species (Paray et al., 2020).

Numerous studies have demonstrated the efficacy of lavender oil in inhibiting the growth of key fish pathogens, including *Aeromonas hydrophila*, *Streptococcus iniae*, *Vibrio anguillarum*, and *Edwardsiella tarda*, bacteria responsible for severe infections such as hemorrhagic septicemia, vibriosis, and streptococcosis in aquaculture systems (Baba et al., 2016; Abdel-Tawwab, 2012; Rattanachai-kunsopon & Phumkhachorn, 2010; Hajirezaee et al., 2022).

The antimicrobial mechanism of lavender oil is primarily linked to its ability to disrupt bacterial cell membranes, leading to increased permeability and leakage of intracellular contents, ultimately resulting in bacterial cell lysis (Wimalasena et al., 2018; Yousefi et al., 2020).

Lavender has also been recognized for its antiparasitic potential, particularly against ectoparasites and endoparasites affecting fish health (Rattanachaikunsopon & Phumkhachorn, 2010; Abdel-Rahim et al., 2024). Studies indicate that lavender oil effectively reduces parasite load in fish infected with *Ichthyophthirius multifiliis* (commonly known as ich), *Gyrodactylus* spp., and *Dactylogyrus* spp., which are major contributors to high mortality rates in aquaculture (Hajirezaee et al., 2022). The anti-parasitic effects of lavender are linked to its bioactive compounds, which interfere with parasite metabolism and disrupt their life cycle, thereby reducing infestation rates and improving fish survival (Moon et al., 2006; Abdel-Tawwab, 2012).

In addition to its direct antimicrobial and antiparasitic effects, lavender supplementation has been shown to enhance fish immune defenses, further contributing to pathogen resistance (Yousefi et al., 2020). By stimulating immune parameters such as lysozyme activity, respiratory burst activity, and complement system activation, lavender strengthens fish resilience against infections and improves overall aquaculture productivity (Baba et al., 2016; Abdel-Rahim et al., 2024).

Given the increasing concerns about antibiotic resistance and chemical residues in aquaculture, lavender essential oil represents a viable and eco-friendly alternative for managing bacterial, fungal, and parasitic

infections. Its broad-spectrum antimicrobial action, combined with its immunostimulatory effects, highlights its potential for sustainable disease management in fish farming (Hoseini & Yousefi, 2019; Hajirezaee et al., 2022).

Beyond its antibacterial properties, lavender also exhibits antifungal effects against common aquaculture-related fungal pathogens, such as *Saprolegnia* spp. and *Aspergillus* spp., which are responsible for skin and gill infections in fish (Hoseini et al., 2020; Yousefi et al., 2019). The antifungal activity of lavender is attributed to its capacity to inhibit fungal spore germination and mycelial growth, preventing the spread of infections within aquaculture systems (Paray et al., 2020).

Effects of lavender on growth performance and feed utilization

Several studies have reported improvements in growth performance, feed utilization, and intestinal morphology in fish supplemented with lavender oil (Abdel-Rahim et al., 2024). Lavender's ability to enhance digestive enzyme activity (amylase, protease, lipase), improve feed conversion ratio (FCR), and increase specific growth rate (SGR) makes it a viable natural growth promoter in aquaculture (Hedayati et al., 2019).

Growth performance enhancement

Fish growth performance is commonly assessed through parameters such as weight gain (WG), specific growth rate (SGR), and condition factor (CF) (Rahimnejad et al., 2021). Studies have reported that dietary supplementation with lavender can positively influence these parameters in various fish species.

Weight gain and specific growth rate

Lavender has been shown to enhance weight gain and specific growth rate in different fish species. In a study by Hassanalizadeh et al. (2020), *Oncorhynchus mykiss* fed diets containing LEON at concentrations of 0, 100, 150, and 200 ml/kg over a 60 days. The results demonstrated that fish receiving 150 ml/kg of LEON exhibited the highest weight gain percentage (WG%) and specific growth rate (SGR), with WG% reaching 252.16%. Similar findings were reported by Al-Derawi et al. (2021), where *Oreochromis niloticus* supplemented with 2% lavender extract demonstrated significant growth improvements due to enhanced protein synthesis and metabolic regulation.

The mechanisms underlying these improvements are attributed to the bioactive compounds in lavender, particularly linalool and rosmarinic acid, which modulate digestive enzyme activity and gut microbiota composition (Mahmoud et al., 2022). This, in turn, facilitates better nutrient absorption and utilization, contributing to enhanced growth performance (Soltani et al., 2021).

Influence on digestive enzyme activity

One of the primary mechanisms through which lavender enhances feed utilization is by modulating digestive enzyme activity. Ashraf et al. (2023) demonstrated that dietary lavender oil supplementation significantly increased the activities of digestive enzymes such as amylase, lipase, and protease in European seabass, leading to improved digestion and nutrient absorption.

Amylase, protease, and lipase activity

Research conducted by Soltani et al. (2021) demonstrated that *Salmo salar* supplemented with lavender essential oil exhibited a 20% increase in amylase and protease activity, facilitating more efficient carbohydrate and protein breakdown. Similarly, Mahmoud et al. (2022) observed a significant upregulation of lipase activity in *Clarias gariepinus*, enhancing lipid metabolism and energy utilization.

These findings align with those of Al-Derawi et al. (2021), who suggested that the phenolic compounds in lavender interact with gut microbiota, promoting the secretion of digestive enzymes. Consequently, fish can extract more energy from their feed, translating into improved growth performance and feed efficiency (Raslan et al., 2025).

Impact on gut microbiota and nutrient absorption

The gut microbiome plays a crucial role in nutrient digestion, immune function, and overall fish health. Recent studies indicate that lavender supplementation can positively modulate gut microbiota composition,

improving nutrient absorption and feed efficiency (Sutili et al., 2018).

Modulation of gut microbiota

In a study by Torrecillas et al. (2021), *Sparus aurata* fed essential oil-supplemented diets exhibited an increase in beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, while pathogenic bacteria like *Aeromonas* and *Pseudomonas* were significantly reduced. This shift in microbial composition enhances gut health and improves the bioavailability of nutrients, leading to better growth performance (Paritova et al., 2024; Ashraf et al., 2023).

Lavender's antimicrobial properties help maintain gut integrity, reducing inflammation and improving nutrient assimilation (Rahimnejad et al., 2021).

COMPARATIVE ANALYSIS OF LAVENDER WITH OTHER LAMIACEAE MEDICINAL PLANTS IN AQUACULTURE

The use of phytogenic additives in aquaculture has gained significant attention due to their ability to enhance fish growth, feed efficiency, immune response, and overall health. Among these, medicinal plants from the Lamiaceae family, including lavender (*Lavandula angustifolia*), thyme (*Thymus* spp.), oregano (*Origanum vulgare*), basil (*Ocimum basilicum*), and rosemary (*Rosmarinus officinalis*), have been extensively studied for their bioactive properties. While lavender has demonstrated substantial efficacy in promoting fish growth and feed utilization, a comparative evaluation with other *Lamiaceae* species is essential to understand its relative benefits.

Lavender vs. Thyme (*Thymus vulgaris*)

Thyme, known for its antimicrobial, antioxidant, and digestive enzyme-enhancing properties, primarily due to its high content of thymol and carvacrol (Rahimnejad et al., 2021; Fathi et al., 2022). Studies have demonstrated its efficacy in improving feed conversion ratio (FCR) and specific growth rate (SGR) in several fish species. For instance, dietary thyme supplementation in *Oncorhynchus mykiss* improved weight gain (WG) by 14%, but a similar concentration of lavender resulted in slightly higher protein efficiency ratio (PER) and better lipid metabolism, suggesting superior nutrient utilization (Yousefi et al., 2022). Thyme has also been found to enhance intestinal microbiota by increasing *Lactobacillus* populations, though lavender has demonstrated a more potent influence on gut health and digestion (Akbari et al., 2023).

Lavender vs. Oregano (*Origanum vulgare*)

Oregano is another widely used phytogenic additive due to its high concentration of polyphenols and essential oils with antimicrobial and digestive-enhancing properties (Leyva-López et al., 2017). Studies on *Sparus aurata* have shown that dietary oregano can improve gut health and increase digestive enzyme secretion (Magouz et al., 2022). However, comparative studies indicate that lavender supplementation yields slightly better nutrient assimilation and antioxidant enzyme activity, possibly due to its higher rosmarinic acid content, which aids in oxidative stress reduction and metabolic balance (Georgiev et al., 2009). Furthermore, oregano has shown notable antibacterial effects against *Aeromonas hydrophila*, but lavender appears to provide broader antimicrobial protection, covering a wider range of pathogens (Alam et al., 2022).

Lavender vs. Basil (*Ocimum basilicum*)

Basil is known for its immunomodulatory and digestive enzyme-stimulating properties, attributed to its high content of flavonoids and essential oils such as eugenol and linalool (El-Bahr et al., 2020). Research by Sharifian et al. (2023) indicated that dietary supplementation with basil significantly enhanced amylase and protease activity in *Oncorhynchus mykiss*, leading to better carbohydrate and protein digestion. However, a comparison with lavender showed that while both plants significantly improved feed utilization, lavender had a greater impact on lipid metabolism and gut microbiota modulation, suggesting superior energy utilization (Karami et al., 2023).

Lavender vs. Rosemary (*Rosmarinus officinalis*)

Rosemary is widely recognized for its potent antioxidant properties, primarily due to its high levels of rosmarinic acid and carnosic acid

(Loussouarn et al., 2017; Naiel et al., 2020). Studies have shown that dietary rosemary enhances fish immunity, reduces oxidative stress, and improves feed utilization in species such as *Cyprinus carpio* and *Salmo salar* (Chelemal et al., 2022). In a study on tilapia, rosemary supplementation improved FCR by 16%,while lavender exhibited comparable effects with additional benefits on gut microbiota composition, reducing pathogenic bacterial loads and increasing beneficial microbial populations (Yilmaz et al., 2019). These findings suggest that lavender not only improves antioxidant capacity but also enhances digestive function and nutrient absorption to a greater extent than rosemary (Sivamaruthi et al., 2024).

Mechanisms responsible for the efficacy of lavender compared to other *Lamiaceae* plants

The superior effects of lavender on fish growth and feed efficiency may be attributed to its unique combination of bioactive compounds, including linalool, rosmarinic acid, and flavonoids (Abdel-Rahim et al., 2024). These compounds not only enhance digestive enzyme activity but also improve gut health by promoting beneficial bacteria while reducing pathogenic microbes (Sutili et al., 2018). Compared to other *Lamiaceae* species, lavender has been shown to exert stronger antimicrobial effects on gut microbiota, leading to better nutrient assimilation and reduced inflammation (Rahimnejad et al., 2021).

Furthermore, lavender's ability to mitigate oxidative stress through its antioxidant properties allows for more efficient energy allocation towards growth rather than stress responses (Fathi et al., 2022). This is particularly evident when compared to rosemary, which, despite its strong antioxidant activity, does not exhibit the same level of enhancement in digestive enzyme activity and gut microbiota modulation (Sharifian et al., 2023).

WAYS OF INTRODUCING LAVENDER INTO FISH DIETS

The incorporation of lavender into fish diets has been widely studied due to its potential health benefits, particularly its immunostimulatory and antimicrobial properties. Different forms of lavender can be used as dietary supplements in aquaculture, including lavender essential oil, dried lavender flowers, aqueous or ethanolic extracts, and nanoemulsions. Each method has distinct advantages in terms of bioavailability, effectiveness, and ease of application (Hajirezaee et al., 2022; Fazio et al., 2017).

Lavender essential oil (LEO)

Lavender essential oil (LEO) is a volatile oil extracted through steam distillation, primarily composed of linalool, linalyl acetate, and camphor, which contribute to its antioxidant and antimicrobial properties (Bajpai et al., 2020). Essential oils have been successfully incorporated into fish diets to enhance immune responses and growth performance (Elumalai et al., 2022).

Studies indicate that adding LEO at optimized concentrations improves fish health. For example, ElHady et al. (2024) found that dietary supplementation of 1% LEO enhanced growth performance, increased antioxidant enzyme activity, and reduced oxidative stress in Nile tilapia (*Oreochromis niloticus*). Similarly, Kuebutornye et al. (2024) reported improved immune function in common carp (*Cyprinus carpio*) when fed diets containing LEO at concentrations of 0.5-1.5%.

Essential oils, including lavender, exhibit antimicrobial activity against various pathogens such as *Aeromonas hydrophila* and *Vibrio* spp. (Chouhan et al., 2017). Dietary incorporation of LEO at 2% significantly reduced bacterial infections in rainbow trout (*Oncorhynchus mykiss*), according to a study by Mirzaei et al. (2023). However, excessive concentrations may cause toxicity and negatively impact fish metabolism (Hosseini et al., 2020), indicating the necessity for dose optimization.

Dried lavender flowers

Dried lavender flowers can be used as a natural supplement in formulated aquafeeds. This method provides a more sustainable and cost-effective alternative to essential oils while still delivering bioactive compounds such as polyphenols, flavonoids, and tannins (Caser et al., 2023). Unlike essential oils, which may lose volatile components over time, dried lavender ensures a gradual release of bioactive

compounds during digestion. Baloochi et al. (2021) reported that feeding dried lavender to rainbow trout enhanced gut microbiota diversity and increased resistance to parasitic infections.

The use of dried lavender also contributes to the sustainable management of aquaculture by utilizing whole plant material, thereby reducing waste. However, proper standardization of drying and processing techniques is essential to maintain bioactive compound integrity (Caser et al., 2023).

Lavender extracts (aqueous and ethanolic)

Lavender extracts, obtained through aqueous or ethanolic extraction, provide highly concentrated bioactive compounds with enhanced bioavailability. Ethanolic extracts, in particular, contain a higher concentration of polyphenols and flavonoids compared to aqueous extracts (Torras-Claveria et al., 2007). These compounds have been linked to immunostimulatory and anti-inflammatory properties in fish (Dobros et al., 2022).

Research by Yousefi et al. (2020) indicated that supplementing fish diets with 2% lavender ethanolic extract significantly enhanced the antioxidant defense system in common carp. Similarly, El-Sayed et al. (2022) found that a combination of lavender aqueous extract and vitamin C improved stress resistance in Asian seabass (*Lates calcarifer*) during transportation. However, the efficacy of lavender extracts depends on their stability and solubility. Encapsulation techniques have been suggested to protect bioactive compounds from degradation during feed processing (Azizi et al., 2021).

Lavender nanoemulsions

Nanoemulsions are emerging as a novel delivery system for lavender bioactive compounds, offering increased absorption and targeted physiological effects. Nanoemulsified lavender essential oil (NE-LEO) enhances bioavailability by improving solubility and protecting active compounds from oxidation (Miastkowska et al., 2023).

Several studies have highlighted the benefits of nanoemulsions in aquaculture. For instance, Rahimi et al. (2023) reported that feeding NE-LEO at 0.3% significantly improved growth rates, gut health, and immune responses in juvenile sturgeon (*Acipenser persicus*). Additionally, nanoemulsions have been shown to reduce stress-induced cortisol levels, as observed in a study on European sea bass (*Dicentrarchus labrax*) by Abdel-Rahim et al. (2024).

Despite their promising applications, the high cost of nanoemulsion technology remains a limiting factor for large-scale implementation. Future research should focus on cost-effective nanoencapsulation techniques and optimizing carrier systems to enhance the stability of lavender-based formulations in aquafeeds (Sheikh et al., 2024).

OPTIMIZING DOSAGE AND ADMINISTRATION METHODS

The efficacy of *Lavandula angustifolia* in fish diets largely depends on the optimal dosage and the form of administration, which influence its bioavailability and physiological effects (Hajirezaee et al., 2022). Various studies have demonstrated that different lavender-derived products, such as essential oils, aqueous or ethanolic extracts, and dried plant material, can positively impact fish health, growth, and immune responses. However, the appropriate dosage varies based on species, life stage, and environmental conditions. Ensuring the correct concentration is crucial to maximizing benefits while preventing potential toxicity, particularly with highly concentrated forms like essential oils (Mirzaei et al., 2023).

Recent research suggests that lavender supplementation improves growth performance, immune response, gut microbiota composition, and oxidative stress resistance across multiple fish species (Dawood et al., 2022). However, the most effective form and dosage depend on species-specific physiological responses and metabolism. The following table summarizes optimal dosages and observed benefits in various fish species (Table 2).

Table 2. Optimal dosages of lavender on different fish species

Fish Species	Optimal Dosage	Observed Benefits	References
Nile Tilapia (*Oreochromis niloticus*)	1-2% extract	Enhanced immunity, improved growth	Al-Deraw et al., 2021
Rainbow Trout (*Oncorhynchus mykiss*)	0.5-1.5% essential oil	Reduced oxidative stress, enhanced antioxidant enzyme activity	Mirzaei et al., 2023
Common Carp (*Cyprinus carpio*)	1-1.5% extract	Improved gut microbiota, increased disease resistance	Rahmanet al., 2021
European Seabass (*Dicentrarchus labrax*)	0.5-1% extract	Enhanced growth performance, reduced lipid peroxidation	Abdel-Rahim et al., 2024
Catfish (*Clarias gariepinus*)	1-2% dried leaves	Improved hematological parameters, reduced stress biomarkers	Ebrahimi et al., 2023
Zebrafish (*Danio rerio*)	0.3-0.7% essential oil	Enhanced antioxidant defense, neuroprotective effects	Sharma et al., 2022
Japanese Flounder (*Paralichthys olivaceus*)	0.8-1.5% extract	Increased survival rates, improved immune gene expression	Liu et al., 2023

Despite these promising findings, further research is needed to standardize optimal dosage recommendations and investigate potential interactions with other phytogenics. Additionally, long-term studies should assess the safety and efficacy of continuous lavender supplementation, ensuring that its benefits persist over time without adverse effects (Sousa et al., 2023).

Potential risks and limitations of lavender supplementation in aquaculture

Lavender (*Lavandula angustifolia* Mill.) has shown promise as a phytoadditive in aquaculture due to its antimicrobial, immunostimulatory, and growth-promoting properties (Smith et al., 2021; Zhao et al., 2022). However, despite these benefits, several limitations and potential risks must be addressed before its widespread implementation in aquafeeds. Excessive doses may result in mild toxicity, metabolic disruptions, alterations in gut microbiota, and challenges related to standardization and quality control (Patel et al., 2021). A thorough evaluation of these factors is essential to optimize the application of lavender in fish nutrition.

Potential toxicity and metabolic disruptions

Lavender contains bioactive compounds such as linalool, linalyl acetate, and rosmarinic acid. However, excessive intake may induce toxic effects in fish species. High concentrations of essential oils and plant extracts have been shown to disrupt metabolic pathways, leading to hepatotoxicity, nephrotoxicity, or oxidative stress (Lis-Balkin et al., 2002; Li et al., 2022). For example, a study on Nile tilapia (*Oreochromis niloticus*) revealed that dietary inclusion of lavender oil above 1% increased hepatic enzyme activity, suggesting metabolic stress and potential hepatotoxic effects (Rodriguez et al., 2020). Similarly, excessive exposure to plant-derived essential oils can induce oxidative damage in fish tissues due to the generation of reactive oxygen species (ROS), impairing normal physiological functions (Hassan et al., 2023).

These findings highlight the necessity of dose optimization to prevent unintended metabolic disturbances while maximizing the benefits of lavender supplementation (Chakraborty et al., 2022).

Effects on gut microbiota and digestive processes

Another concern associated with lavender supplementation is its potential to alter gut microbiota composition in fish, which may affect nutrient absorption and immune function (Navarro et al., 2022). The gut microbiome plays a crucial role in fish health, aiding digestion, pathogen resistance, and immune modulation (Merrifield & Dimitroglou, 2021). However, excessive phytochemical intake may disrupt microbial equilibrium, favoring the proliferation of certain bacterial taxa while suppressing beneficial species (Wang et al., 2023).

Such imbalances may compromise gut integrity, leading to digestive inefficiencies and increased susceptibility to infections. Phytochemicals from lavender, particularly polyphenols, may exert antimicrobial effects that inadvertently suppress beneficial gut bacteria required for optimal digestion and immunity (Feng et al., 2021).

Digestive enzyme activity may also be affected by lavender supplementation. Research on Asian seabass (*Lates calcarifer*) indicated that lavender extract at high concentrations (above 4 g/kg) reduced amylase and protease enzyme activity, potentially interfering with nutrient assimilation (Das et al., 2020). While lavender provides immunostimulatory advantages, its impact on digestive physiology necessitates careful formulation strategies to avoid negative effects on gut health and nutrient utilization (Rahman et al., 2021).

CHALLENGES IN STANDARDIZATION AND QUALITY CONTROL

One significant challenge in utilizing lavender as an aquafeed additive is ensuring consistency in efficacy and safety across different batches of feed formulations. The bioactive composition of lavender extracts can vary considerably depending on plant cultivar, growth conditions, extraction methods, and storage conditions (Gupta et al., 2021). This variability poses a challenge in achieving standardized dosing, which is critical for maintaining predictable biological responses in fish (Karakaya et al., 2022).

For example, a comparative study on different lavender cultivars demonstrated substantial variation in linalool and rosmarinic acid content, with some extracts exhibiting significantly higher antioxidant activity than others (Silva et al., 2022). Such inconsistency raises concerns regarding the reproducibility of research findings and the effectiveness of lavender-based feed additives in commercial aquaculture settings (Pavlidis et al., 2023). Moreover, standardization issues may lead to fluctuating immune responses, as variations in phytochemical concentrations could result in either suboptimal or excessive immune stimulation, thereby affecting fish health outcomes (Castro et al., 2021).

Potential contamination with heavy metals, pesticides, or fungal toxins during lavender cultivation and processing poses another safety risk (Popescu et al., 2019; Ferreira et al., 2022). Quality control measures such as chromatographic fingerprinting and bioassays are essential to ensure that lavender-based additives meet required safety and efficacy standards (Gonzalez et al., 2021). Addressing these challenges will be crucial for the successful integration of lavender into sustainable aquafeed formulations.

Implications for commercial application

Given the potential risks associated with lavender supplementation, it is imperative to establish appropriate guidelines for its inclusion in aquaculture diets. Studies suggest that safe dosage levels vary among fish species, with most research indicating optimal effects at concentrations between 0.5% and 1.5% of total feed weight (Jiang et al., 2022). However, exceeding these thresholds may lead to adverse metabolic, microbial, or toxicological effects, underscoring the importance of species-specific trials and regulatory oversight (Martinez et al., 2023).

Furthermore, synergistic effects between lavender and other phytoadditives should be explored to optimize its benefits while reducing potential limitations. For example, combining lavender with prebiotics or probiotics may help maintain gut microbial balance, preventing dysbiosis while enhancing immune responses (Alvarez et al., 2022). Similarly, encapsulation technologies could improve the bioavailability and controlled release of lavender bioactives, reducing the risk of toxicity and metabolic disruptions (Oliveira et al., 2021).

CONCLUSIONS

Lavender is a promising phytoadditive in aquaculture, enhancing fish health, growth, and immunity through its bioactive compounds, particularly linalool and flavonoids, which improve nutrient absorption, gut microbiota, and oxidative stress resistance. Different administration methods, such as essential oils, dried flowers, and extracts, offer distinct benefits, with nanoemulsions enhancing bioavailability but requiring higher production costs. Future research should focus on optimizing dosage, evaluating long-term effects, and exploring synergies with other phytogenics across different fish species, life stages, and environmental conditions. Compared to other Lamiaceae plants, lavender demonstrates superior impacts on protein metabolism, gut microbiota, and oxidative stress reduction. Consideration of dosage and

potential risks is necessary to ensure its safe and effective integration into sustainable aquaculture.

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BLOOD PARAMETERS OF RAINBOW TROUT *Oncorhynchus mykiss* (Walbaum, 1792) FED DIETS SUPPLEMENTED WITH NATURAL PHYTOADDITIVES DURING THE COLD SEASON

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Abstract

The use of phytoadditives in animal feeds has been gaining more attention due to their positive effects on growth and health of animals. This study aimed to determine the effects of adding natural phytoadditives to the diet of rainbow trout on its blood parameters during the cold season. One control and three experimental groups consisting of 50 adult rainbow trout each were fed a standard feed and three experimental diets consisting of the standard feed with 2% carrot, tomato, and spinach powders, respectively, for 90 days. Blood parameters were determined. Most parameters changed significantly ($p<0.05$) from the start to the end of the experiment. There were few significant changes among the control and experimental groups at the end of the experiment. Cholesterol and triglycerides increased in the control and experimental group with 2% carrot powder. Some minerals showed different ($p<0.05$) values in the experimental groups (Na, K, and Ca). The study shows that the incorporation of phytoadditives in rainbow trout feed does not produce negative effects on the blood parameters, with some advantages being present, such as a generally better mineral profile of the plasma and the stabilization of hematological parameters.

Key words*: carrot, hematology, plasma biochemistry, salmonids.*

INTRODUCTION

Natural phytoadditives have gained more and more interest for aquaculture due to their potential benefits for fish health, growth performance, and disease resistance (Ivan et al., 2022; Kuebutornye et al., 2023; Ivan et al., 2024a; Zhelyazkov & Stoev, 2024). Flavonoids, carotenoids, saponins, and other compounds found in different plants have antimicrobial, antioxidant, and immune stimulatory properties (Samtiya et al., 2021; Rahimi et al., 2022; Hu et al., 2024). Thus, phytoadditives can serve as alternatives to synthetic antibiotics, reducing the risk of antibiotic resistance, minimizing environmental contamination and presenting a safer product for consumers (Păpuc et al., 2024). There are many studies demonstrating that dietary supplementation with plant extracts improves growth performance and immunity in cultured species (Gherescu et al., 2023).

Consumer awareness regarding the welfare of farmed animals, including fish, has been continuously increasing, especially based on access to transparent information about farming practices. Certification programs and labelling schemes have been developed to address ethical concerns about aquaculture (Hammarlund et al., 2024). Farmers must consider the nature of the feed used, as natural ingredients in fish feed may prove more attractive and safer for consumers concerned with the welfare of the fish and with the sustainability of the practice. Thus, the supplementation of fish feeds with

phytoadditives could not only provide benefits for the fish, but also for the farmers, in the form of a distinct selling point.

Classic intensive salmonid farms need a careful management of environmental parameters. Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) is more resistant to fluctuations in these factors than other salmonids. However, they still directly impact growth rates and overall health (Uiuiu et al., 2021). Climate variability and seasonal changes pose significant challenges to rearing conditions (Tekeli & Bildik, 2023). Rainbow trout experience seasonal variations in growth, with stagnation or reduction in winter due to lower metabolic activity (Tidwell et al., 1991; Taylor et al., 2006). Water temperature is a primary determinant of growth, as metabolic processes are reduced in colder temperatures, lowering feed intake and weight gain, energy conservation becoming a priority. Consequently, aquaculture systems must adapt feeding strategies to accommodate these physiological changes during the winter.

Many traditional trout farms adopt a winter fasting strategy, drastically decreasing feed administration, or even ceasing it during the coldest months. This practice aligns with the reduced metabolic rate of trout, helping prevent feed waste. Winter fasting has some advantages, such as the reduction of operational costs or minimization of uneaten feed accumulation, but also some disadvantages, like the possibility of excessive weight loss or fish health deterioration (Weatherup et al., 1997). Therefore, the decision to implement fasting should consider these factors. On the other hand, some farmers prefer to adopt different strategies, such as feeding to satiation or the administration of sufficient feed to maintain trout weight during winter (Taylor et al., 2006). However, the effects of such feeding strategies during winter on rainbow trout health are less studied.

Blood indices provide essential information about the physiological and health status of rainbow trout. Hemoglobin concentration, erythrocyte count, hematocrit levels, plasma glucose, and enzymatic activity serve as indicators of stress, nutritional status, and immune function (Cocan et al., 2019). Multiple factors simultaneously influence blood parameters in rainbow trout, including water temperature, stocking density, handling stress, age, sex of the fish, and many others (Barnhart, 1969; Martinez et al., 1994; Yarahmadi et al., 2014; Jiang et al., 2021; Manera et al., 2021). Seasonal changes, particularly winter conditions, can alter hematological and biochemical profiles due to metabolic adaptations (Lahnsteiner & Dünser, 2024). Monitoring blood parameters allows the assessment of the diet, environmental parameters, and disease impacts on fish physiological status. Understanding these interactions is crucial for optimizing trout farming technologies and improving fish welfare in aquaculture systems.

The present study aims to assess the blood parameters of rainbow trout fed with phytoadditive-supplemented feeds, in a classic, intensive trout farm, in the cold season.

MATERIALS AND METHODS

The present study was conducted at a salmonid farm, near Cluj-Napoca, Romania, from November 2023 to February 2024, for 90 days. From an initial group (Gi) of 200 fish, the control and the three experimental groups were formed, each with 50 fish, consisting of clinically healthy adult rainbow trout. Each group was assigned to a race-way type concrete pond, with a volume of 2.5 m^3 (5 x 1 x 0.5 m), being stocked at a density of 20 ind m^{-3}.

A standard feed in the form of 6 mm pellets was used as the control feed, for the control group (G1). The experimental feeds consisted of the standard feed with an addition of 2% meal from different natural carotenoid sources: carrots (Nantes), tomatoes (giant tomato) and spinach (Nores). The vegetal materials were previously farmed in a nearby household, harvested, dried in an aerated oven at 39.5°C, and blended to obtain a powder from each type of material used. Three types of experimental feed were prepared, each with a 2% addition of one of the vegetal powders, obtaining an experimental feed with 2% carrot powder (EFC) fed to group G2, an experimental feed with 2% tomato powder (EFT) fed to group G3, and an experimental feed with 2% spinach powder (EFS) fed to group G4. The phytoadditive was added after mixing with water, simply by coating the pelleted feed and

drying in a ventilated oven at 39.5ºC. The feed was administered once per day, until satiation.

The chemical composition of the powders and of the experimental feeds is presented in Table 1. The proximate composition of the feeds and powders was determined using the FT-NIR Tango from Bruker (Billerica, MA, USA) (Masithoh et al., 2020). Five samples were selected from each studied material, and each sample suffered triplicate measurements.

Table 1. Chemical composition of phytoadditives and experimental feed

Phytoadditives and feeds	DM (%)	Protein (%)	Fat (%)	Ash (%)	Fibers (%)
Carrot powder	81.96±0.84	8.48±0.1	1.64±0.09	1.32±0.05	36.46±0.92
Tomato powder	84.3±0.5	15.69±0.43	1.96±0.18	2.85±0.07	22.3±0.82
Spinach powder	75.54±1.12	13.73±0.12	2.69±0.12	3.12±0.06	32.31±0.79
Standard feed	91.53±0.57	37.82±0.47	13.76±1.29	5.27±0.03	5.88±1.29
EFC	91.53±0.2	36.67±0.47	13.49±1.28	5.19±0.04	6.21±0.49
EFT	91.54±0.29	36.85±0.46	13.56±1.24	5.21±0.02	5.93±0.48
EFS	91.44±0.16	36.59±0.51	13.53±1.24	5.22±0.02	5.99±1.21

EFC - experimental feed with 2% addition of carrot powder; EFT - experimental feed with 2% tomato powder addition; EFS - experimental diet with 2% spinach powder addition; DM - dry matter; values are presented as means ± SD (n = 5).

Water parameters were specific to an intensive salmonid farm, with outside ponds, in a temperate climate region, during the cold season. At the start of the experiment, the temperatures had the highest values, decreasing until the end of the experiment. The water source was the same for each pond, a drinking water supply reservoir, situated above the farm. The water flow was maintained at 3 L s^{-1} during the experimental period. The water parameters determined were: temperature, dissolved oxygen (DO), pH, electrical conductivity, total dissolved solids (TDS), and turbidity. The water parameters were monitored daily, before the first feeding, with a Hanna HI 9829 Multiparameter, as water quality is an essential factor in the success of a trout farm (Ivan et al., 2024b). The water having the same source, its parameters did not suffer differences among ponds. The water parameters are presented in Table 2.

Table 2. Water parameters during the study

Descriptive statistics parameters	Parameters					
(n = 90)	Temp.	DO	pH	Electrical conductivity	TDS	Turb. [FNU]
Mean	5.6972	14.0084	6.823	78.6111	38.9222	1.0633
SD	2.368	0.6045	0.1231	3.4502	1.8512	0.6138
Min.	2.74	12.18	6.6	72	36	0.2
Max.	9.91	14.98	7.12	86	43	2.6

SD - standard deviation; Temp - temperature; DO - dissolved oxygen; TDS - total dissolved solids; Turb. - turbidity.

Blood samples were randomly collected from 10 rainbow trout at the start of the experiment, from the initial group Gi, of 200 fish. Five samples were used for hematological analyses and five others for plasma biochemical analyses, as the volume did not allow to perform all the analyses from a single sample. At the end of the experiment, samples were collected similarly from ten trout, this time from each of the four groups. Blood was collected by puncture of the caudal vein and transferred to vacutainers with LiHeparin for analyses. The fish were priorly anesthetized with clove oil at a dose of 30 mg L^{-1} (Javahery et al., 2012). As anaesthesia can influence blood parameters, care was taken to keep the anaesthesia time to a minimum. The blood samples were transported in refrigerated conditions (2-4°C) to the Hematology and Biochemistry Laboratory of UASVM Cluj-Napoca, for further analyses (Cocan et al., 2018). After the blood samples were collected, the sampled fish were transferred to well-oxygenated water to facilitate recovery.

The hematological profile was characterized by determining hemoglobin (Hb), erythrocytes (Ery), and hematocrit (Hct) from the blood samples. The Hb concentration was determined within a maximum of 6 h after harvesting by the END-POINT method, employing a colorimetric reaction, with VIS reading at 546 nm and 37°C. Erythrocyte determinations were conducted using Gowers reagent through a turbidimetric

reaction in the visible range at a wavelength of 546 nm and at 37°C. The Hct was determined by centrifugation in capillary tubes at 12000 rotations per min for 3 min. The Ery indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to standard formulas (Sarma, 1990).

All analyses were performed per the instructions of commercial test kits available for each parameter with the UV-VIS Screen Master Touch spectrophotometer in the 340-620 nm wavelength range.

The protein profile parameters determined were the following: total protein (TP) concentration, albumins (ALB), gamma globulins (GG), urea (UR), and creatinine (CRN). TP was determined at 546 nm in the VIS domain and ALB at 630 nm by the END-POINT type method. The GG concentration was determined turbidimetrically at 546 nm in the VIS domain and UR at 340 nm with the kinetic enzymatic method. CRN was determined by a fixed-time colorimetric method (FXT) with VIS reading at 510 nm.

To analyse the lipid profile, the concentrations of triglycerides (TG) and cholesterol (CHOL) were determined through END-POINT type colorimetric reaction at 546 nm (TG) and 510 nm (CHOL).

The following enzymes were determined for the enzymatic profile: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma-glutamyltransferase (GGT), and lactate dehydrogenase (LDH). ALAT, ASAT, and LDH were determined at 340 nm by the kinetic method. GGT was determined at 405 nm.

The following parameters were analysed for the mineral profile: Ca^{2+}, PO_4^{3-}, Fe^{3+}, Na^{+}, Mg^{2+} and K^{+}. The Ca^{2+}, PO_4^{3-}, Mg^{2+} and Fe^{3+} determinations were made using an END-POINT-type colorimetric reaction at 650 nm (Ca^{2+}), 340 nm (PO_4^{3-}), 405 nm (Fe^{3+}), and 510 nm (Mg^{2+}). Na^{+} was made using the colorimetric enzyme type method. K^{+} was determined by the kinetic method at 380 nm.

Glucose (GLC) was determined through the END-POINT enzymatic colorimetric assay (λ=500 nm). Total bilirubin (TB) was also determined, at 540 nm.

Data analysis was performed using GraphPad Prism 9 software. The Shapiro-Wilk test was used to assess the normality of data distribution. When data followed a normal distribution, differences among groups were analysed using one-way ANOVA, followed by Tukey's post-hoc multiple comparison test. For data that did not meet the assumption of normality, the Kruskal-Wallis test was applied, followed by Dunn's multiple comparison test. The significance level was set at $\alpha=0.05$.

RESULTS AND DISCUSSIONS

The hematological profile presented no significant differences ($p>0.05$) for the studied parameters among G1 and the experimental groups. However, there were significant differences ($p<0.05$) for some parameters between the start and the end of the experiment (Table 3).

The obtained Hb values are relatively normal considering the low average water temperature and high DO levels during the study. At higher temperatures, Martinez et al. (1994) obtained Hb values between 6.2 and 12.1 g dL^{-1}. Erythrocyte levels ranged from 2.66 to 3.41x106 mm^{-3}, a range considered normal by some authors (Řehulka et al., 2004; Nabi et al., 2022; Lahnsteiner & Dünser, 2024), but higher than normal by others (Mesalles et al., 2024).

Table 3. Hematological profile of rainbow trout

Group	Hb (g dL^{-1})	Ery (x 10^6 mm^{-3})	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})
Gi	5.88±1.16^{a}	2.66±0.3^{a}	30.4±7.6^{a}	112.85±15.57^{a}	21.99±2.68^{a}	19.65±2.72^{a}
G1	6.31±0.49^{a}	3.41±0.33^{a}	45.6±5.41^{b}	133.77±9.56^{ab}	18.58±1.22^{a}	13.95±1.39^{b}
G2	6.47±0.58^{a}	2.97±0.36^{a}	43.6±7.43^{ab}	145.83±11.75^{b}	21.82±1.3^{a}	15.07±1.82^{b}
G3	6.45±1.03^{a}	3.25±0.42^{a}	43.2±7.94^{ab}	132.22±10.31^{ab}	19.95±3.54^{a}	15.23±3.43^{b}
G4	6.49±1.29^{a}	3.08±0.56^{a}	44.2±9.03^{ab}	143.44±14.86^{b}	20.98±2.17^{a}	14.66±1.1^{b}

Gi - initial group; G1 - control group; G2 - group fed with 2% addition of carrot powder in the feed; G3 - group fed with 2% addition of tomato powder in the feed; G4 - group fed with 2% addition of spinach powder in the feed; Hb - hemoglobin; Ery - erythrocytes; Hct - hematocrit; MCV - mean cell volume; MCH - mean cell hemoglobin; MCHC - mean corpuscular hemoglobin concentration; values are presented as mean ± SD (n = 5); different superscripts in the same column show significant differences ($p<0.05$); Hb, Hct, MCV, MCH and MCHC data were analysed with one-way ANOVA, followed by Tukey's multiple comparisons test; Ery data was analysed with the Kruskal-Wallis test.

The Hct had values that can be considered upper normal (Wells & Weber, 1991). A clear increase was observed at the end of the experiment, with a peak of 45.6% in the control group. These slightly elevated values can be attributed to the lower temperatures of the water at the end of the experiment.

MCV values increased by the end of the experiment in all groups, with significant differences ($p<0.05$) between MCV levels in Gi, and G2 and G4. As the optimal MCV level is between 350 and 400 fL (FAO, 1991), the values obtained in this study can be considered low for rainbow trout, indicating small sizes for erythrocytes. There are instances with a lower MCV, for example, when rainbow trout is exposed to zinc or cobalt chloride (Atamanalp et al., 2011; Baladehi et al., 2017). Fazio et al. (2016) note that healthy rainbow trout in an Italian farm had an MCV of 190.8 fL, while healthy rainbow trout in a Turkish farm had a mean MCV of 85.66 fL. Ihuț et al. (2018) also note a decrease of MCV in winter for rainbow trout, with a value of 109.62 ± 3.17 fL. MCH values were situated between 18.58 and 21.99 pg. Although normal MCH values for rainbow trout are higher than the values obtained in the current study (Fazio et al., 2017a; Ihuț et al., 2018), in some cases, comparable values were obtained for healthy rainbow trout (Lone et al., 2012; Fazio et al., 2016). The MCHC values obtained in this study at the end of the experiment are within the normal range (8.8-18.2 g dL^{-1}) according to Mesalles et al. (2024), while the initial value slightly exceeds the normal range. Most studies find MCHC in healthy rainbow trout at higher values (Fazio et al., 2017b; Ihuț et al., 2018; Nabi et al., 2022), although there are some reports similar to the results of this study (Řehulka et al., 2004).

As the fish experienced normal conditions in the winter season in an intensive salmonid farm, without significant differences between the experimental groups and the control group regarding the hematological profile, the most likely explanation for values outside normal ranges may be cold-induced physiological adaptations.

Generally, the protein profile of the plasma remained the same throughout the experiment. There were two cases when significant differences occurred, namely: the TP concentration in G2 was significantly higher ($p<0.05$) than that of G4, and the CRN level in Gi was significantly higher ($p<0.05$) than that of fish from G1. The protein profile obtained is presented in Table 4.

TP levels in the plasma of rainbow trout obtained in this study were relatively normal. Řehulka et al. (2004b) found TP levels between 3.1-5.8 g dL^{-1}, with significant differences between males and females, while Manera & Britti (2006) suggest a normal level of TP in rainbow trout of 3.32-3.85 g dL^{-1}. TP generally shows the health status of the fish. Morro et al. (2020) identified 1822 proteins in the plasma of rainbow trout.

ALB levels were relatively normal, in line with the findings of many different studies (Vigiani et al., 2005; Manera & Britti, 2006; Pastorino et al., 2022). Monitoring albumin levels may help indicate the nutritional status and overall health of rainbow trout.

Table 4. Protein profile of rainbow trout plasma

Group	Total protein concentration (g dL^{-1})	Albumin concentration (g dL^{-1})	Gamma globulin concentration (g dL^{-1})	Urea (mg dL^{-1})	Creatinine (mg dL^{-1})
Gi	4.58 ± 1.62^{ab}	1.44 ± 0.50^{a}	0.63 ± 0.35^{a}	14.94 ± 1.53^{a}	0.65 ± 0.12^{a}
G1	4.63 ± 0.36^{ab}	1.30 ± 0.08^{a}	0.38 ± 0.09^{a}	15.64 ± 2.80^{a}	0.43 ± 0.05^{b}
G2	5.17 ± 0.42^{a}	1.57 ± 0.18^{a}	0.44 ± 0.10^{a}	14.84 ± 0.99^{a}	0.54 ± 0.08^{ab}
G3	4.31 ± 0.13^{ab}	1.29 ± 0.14^{a}	0.27 ± 0.11^{a}	16.18 ± 3.00^{a}	0.47 ± 0.02^{ab}
G4	4.02 ± 0.36^{b}	1.30 ± 0.17^{a}	0.38 ± 0.13^{a}	14.54 ± 1.59^{a}	0.47 ± 0.15^{ab}

Gi - initial group; G1 - control group; G2 - group fed with 2% addition of carrot powder in the feed; G3 - group fed with 2% addition of tomato powder in the feed; G4 - group fed with 2% addition of spinach powder in the feed; values are presented as mean ± SD (n = 5); different superscripts in the same column show significant differences ($p<0.05$); total protein concentration data was analysed with the Kruskal-Wallis test, followed by Dunn's multiple comparisons test; albumin, gamma globulin concentration, urea and creatinine data were analysed with one-way ANOVA followed by Tukey's multiple comparisons test.

GG levels varied from 0.27 to 0.63 g dL^{-1}. GG in rainbow trout are a key indicator of immune health and disease resistance. Olesen & Jørgensen (1986) determined serum immunoglobulin M (IgM) levels in rainbow trout at 0.33 g dL^{-1}, with a minimum of 0.11 and a maximum of 1.06 g dL^{-1}. Aghili et al. (2024) determined globulin levels from 1.9 to 2.92 g dL^{-1}, with increases when phytoadditives (curcuma and black pepper) were added to the feed of rainbow trout. The values obtained in this study are similar to those obtained by Aghili et al. (2024). GGs are also dependent on environmental parameters. Sano (1960) observed a decrease of GG in rainbow trout when temperatures decrease, an observation later confirmed by Le Morvan et al. (1998) for most teleost fish.

Usually, UR levels in rainbow trout are under 1 mg dL^{-1} (Wedemeyer & Chatterton, 1970). UR levels were much higher than normal in the current study. Other studies also determined UR levels in rainbow trout up to 7.21 mg dL^{-1}, in some pathological cases (Řehulka, 1998), or under different environmental conditions (Řehulka, 2000; Kopp et al., 2011). A peak of 25 mg dL^{-1} was found in the case of trout fed a UR supplement (Kaushuk et al., 1983). The high values in this study may have occurred due to metabolic stress because of extremely cold temperatures.

CRN levels can provide insights into renal health and overall physiological status (Banaee et al., 2023). The values obtained in this study are just above normal levels (0.09-0.45 mg dL^{-1}) (Wedemeyer & Chatterton, 1970). It is not clear if temperature directly influences creatinine levels. However, it does impact metabolic processes, suggesting that temperature may indirectly affect metabolic waste products (Kieffer et al., 1994).

The lipid profile of the plasma changed significantly during the experiment. In Gi, the CHOL level was 280.72±95.71 mg dL^{-1}, significantly lower ($p<0.01$) than the value in G1 (555.66±92.65 mg dL^{-1}), and that of G2 (625.16±183.01 mg dL^{-1}; $p<0.001$). Fish from G2 had a CHOL level significantly higher than that of G3 ($p<0.05$) and G4 ($p<0.01$), with 381.28±63.93 and 361.74±63.08 mg dL^{-1}, respectively (Figure 1).

TG levels increased significantly ($p<0.01$) during the experiment in G1 (876.1± 318.63 mg dL^{-1}) compared to Gi (299.78±94.75 mg dL^{-1}). Similarly, the TG levels in G2 (920.02±261.33 mg dL^{-1}) were also significantly higher ($p<0.001$) that those obtained from fish in Gi (Figure 2).

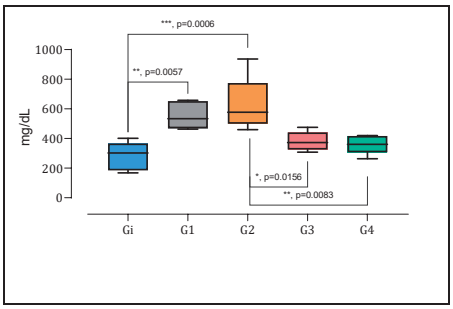


Figure 1. Cholesterol levels of rainbow trout plasma; Gi - initial group; G1 - control group; G2 - group fed with 2% addition of carrot powder in the feed; G3 - group fed with 2% addition of tomato powder in the feed; G4 - group fed with 2% addition of spinach powder in the feed; data was analysed with one-way ANOVA, followed by Tukey's multiple comparisons test

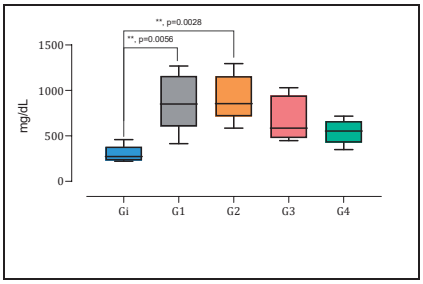


Figure 2. Triglyceride levels of rainbow trout plasma; Gi - initial group; G1 - control group; G2 - group fed with 2% addition of carrot powder in the feed; G3 - group fed with 2% addition of tomato powder in the feed; G4 - group fed with 2% addition of spinach powder in the feed; data was analysed with one-way ANOVA, followed by Tukey's multiple comparisons test

CHOL levels increased in all treatments and in the control, exceeding normal levels of 161-365 mg dL^{-1} (Wedemeyer & Chatterton, 1970) in G1 and G2. TG levels had a similar trend, with levels in G1 and G2 greatly increased.

A plausible explanation for the high levels of lipid indices, and for some plasma protein indices, can be constructed from the following factors: low water temperature, slowing

metabolism activity, and gonadal maturation (unpublished data from the current experiment). Bhat et al. (2022) noticed increased protein and lipid levels during the gonadal maturation in rainbow trout. Other authors also noticed increased lipid and protein levels in salmonid plasma during gonadal maturation (Johnston et al., 1987; Bon et al., 1997; Jenkins et al., 2019). Hoseini et al. (2013) noticed a different factor influencing TG level, namely fasting before blood sampling. In the present study, the increase in photoperiod and a slight increase in water temperature by the end of the experiment could have triggered gonadal development.

The values of the enzymatic profile parameters of the plasma did not differ significantly among the initial results, control and treatments. The values are presented in Table 5.

Table 5. Enzymatic profile of rainbow trout plasma

Group	Alanine aminotransferase (U L^{-1})	Aspartate aminotransferase (U L^{-1})	Gamma-glutamyltransferase (U L^{-1})	Lactate dehydrogenase (U L^{-1})
Gi	6.06±3.84^{a}	130.52±67.24^{a}	5.51±2.47^{a}	967.78±330.28^{a}
G1	3.48±1.55^{a}	97.8±26.8^{a}	5.98±3.15^{a}	326.46±147.29^{a}
G2	11.62±2.34^{a}	96.42±16.08^{a}	7.42±4^{a}	553.32±259.15^{a}
G3	11.08±5.24^{a}	83.66±37.66^{a}	8.8±4.69^{a}	248.24±78.42^{a}
G4	9.06±6.74^{a}	82.84±29.01^{a}	4.55±2.63^{a}	436.68±307.94^{a}

Gi - initial group; G1 - control group; G2 - group fed with 2% addition of carrot powder in the feed; G3 - group fed with 2% addition of tomato powder in the feed; G4 - group fed with 2% addition of spinach powder in the feed; values are presented as mean ± SD (n = 5); different superscripts in the same column show significant differences ($p<0.05$); alanine aminotransferase, and lactate dehydrogenase data were analysed with the Kruskal-Wallis test; gamma-glutamyltransferase, and aspartate aminotransferase data were analysed with one-way ANOVA.

ALAT serves as a useful biomarker for assessing liver health and general metabolic stress in fish. It varies under different conditions, being dependent on feed, environmental conditions, and other factors. In this study, ALAT levels ranged from 3.48 to 11.62 U L^{-1}. Considering the slow lipid metabolism due to low water temperature, the levels can be considered normal. Cao et al. (2023) obtained comparable levels of ALAT in rainbow trout farmed in net cages, ranging from 3 to 8.67 U L^{-1}, while Rozas-Serri et al. (2022) obtained values between 7.44-15.87 U L^{-1} for rainbow trout, depending on life stage, with a mean of 10.33±4.95 U L^{-1} for adults.

ASAT is commonly used as a biomarker for liver and muscle health in fish. As ALAT, ASAT varies based on many factors. Lower levels than those obtained in the current study were reported by Pastorino et al. (2020) for rainbow trout under normal conditions, while a range between 290.74 and 362.46 U L^{-1} was reported by Rozas-Serri et al. (2022), with a mean of 316.66±129.73 U L^{-1} for adult rainbow trout.

GGT is an enzyme involved in the metabolism of glutathione and the transfer of amino acids across cell membranes. Pastorino et al. (2020) determined GG levels in rain-bow trout similar to those obtained in the present study. Usually, GGT levels are low in the plasma of rainbow trout (Bauermeister et al., 1983).

LDH is an enzyme involved in energy production. LDH decreased from the initial values in this study, possibly due to the low water temperature. The LDH values at the end of the experiment were lower than those obtained by Rozas-Serri et al. (2022), who established an interval between 696.93 and 1215.77 U L^{-1} for rainbow trout under normal conditions, with a mean for adults of 882.25±308.17 U L^{-1}. Manera & Britti (2006) obtained a much higher mean value, of 2628.18 U L^{-1}, considering normal ranges of 462.04-4794.31 U L^{-1}.

PO_4^{3-}, Fe^{3+}, and Mg^{2+} did not suffer significant changes ($p>0.05$) during the experiment. The highest value of Ca^{2+} was observed in G2 (13.16±0.82 mg dL^{-1}), while the lowest was observed for G4 (8.56±0.56 mg dL^{-1}). The highest Na^{+} value was obtained in G3 (165.54±4.95 mmol L^{-1}), while the lowest was observed in Gi (141.38±8.08 mmol L^{-1}). The highest K^{+} level was observed in G4 (3.33±0.50 mmol L^{-1}), significantly different ($p<0.05$) from the value observed in G1 (2.33±0.14 mmol L^{-1}). The mineral profile of rainbow trout plasma is presented in Table 6.

Table 6. Mineral profile of rainbow trout plasma

Group	Ca^{2+} (mg dL^{-1})	PO_4^{3-} (mg dL^{-1})	Fe^{3+} (µg dL^{-1})	Na^{+} (mmol L^{-1})	K^{+} (mmol L^{-1})	Mg^{2+} (mg dL^{-1})
Gi	11.55±1.52^{abc}	10.47±3.09^{a}	133.06±19.01^{a}	141.38±8.08^{a}	2.73±0.30^{ab}	3.20±0.59^{a}
G1	11.09±0.72^{ab}	10.38±0.67^{a}	160.44±55.03^{a}	158.04±2.40^{ab}	2.33±0.14^{a}	3.53±0.10^{a}
G2	13.16±0.82^{c}	11.27±1.30^{a}	154.96±24.99^{a}	155.28±10.55^{ab}	2.48±0.38^{ab}	3.74±0.68^{a}
G3	10.92±0.82^{b}	11.71±2.47^{a}	114.24±18.55^{a}	165.54±4.95^{b}	2.77±0.20^{ab}	3.58±0.66^{a}
G4	8.56±0.56^{d}	11.84±0.82^{a}	93.16±34.08^{a}	156.00±2.05^{ab}	3.33±0.50^{b}	3.46±0.31^{a}

Gi - initial group; G1 - control group; G2 - group fed with 2% addition of carrot powder in the feed; G3 - group fed with 2% addition of tomato powder in the feed; G4 - group fed with 2% addition of spinach powder in the feed; values are presented as mean ± SD (n = 5); different superscripts in the same column show significant differences ($p<0.05$); Ca^{2+}, Fe^{3+}, and Mg^{2+} data were analysed with one-way ANOVA, followed by Tukey's multiple comparisons test; PO_4^{3-}, Na^{+} and K^{+} data were analysed with the Kruskal-Wallis test, followed by Dunn's multiple comparisons test.

Calcium is vital for the overall health and proper physiological functioning of rainbow trout. It has roles in bone health, muscle function, nerve signalling and reproductive health. The calcium results from the present study are within the range pro-posed by Řehulka & Minařík (2008), between 10 and 16 mg dL^{-1}, with the exception of the Ca level in G4, which was slightly lower. Rozas-Serri et al. (2022) obtained higher values, with a mean of 53.45±7.86 mg dL^{-1} Ca in the plasma of adult rainbow trout.

In rainbow trout, phosphate is involved in various biochemical processes, including the synthesis of nucleic acids and proteins, and the formation of ATP. Proper phosphate levels are vital for growth, health, and overall physiological functions (Coloso et al., 2003). Usually, phosphate levels in rainbow trout plasma ranges above the values obtained in this study (Řehulka & Minařík, 2008; Kopp et al., 2011; Prabhu et al., 2014; Rozas-Serri et al., 2022). However, it is important to note that phosphate uptake in cold water is impeded, and that energy requirements during the winter season are also lower for rainbow trout, low temperature affecting phosphate metabolism (Kurnianingtyas & Syahidah, 2024). Sugiura (2015) obtained similar levels to those obtained in the present study.

Iron has roles primarily in oxygen transport, enzyme function, and overall metabolism. Kwong et al. (2013) reported levels of 55.9 to 223.4 µg dL^{-1} Fe in rainbow trout fed with a control feed and a feed supplemented with iron, a range including the values obtained in this study. Rainbow trout maintains iron homeostasis through dietary regulation and efficient storage mechanisms (Carriquiriborde et al., 2004). Iron metabolism is also influenced by temperature, decreased temperatures lowering metabolic rates, reducing the need for oxygen transport and potentially decreasing the synthesis of iron-dependent proteins (Kochhann et al., 2024).

Sodium is an essential electrolyte in rainbow trout plasma, playing a crucial role in osmoregulation, acid-base balance, nerve function, and overall metabolic processes. Cold temperatures (2-4°C) can reduce sodium uptake efficiency due to slower gill ion exchange and metabolism. The values obtained in this study are similar to those ob-tained by Hrubec & Smith (1999) in rainbow trout plasma, namely 152 mmol L^{-1}. Sodium levels are influenced by temperature, decreasing in colder waters (Brown et al., 1999).

Magnesium is also an essential electrolyte in rainbow trout plasma, playing an important role in enzyme function, nerve transmission, muscle contraction, osmoregulation, and metabolic balance. The values obtained in this study are on the upper end of the values obtained by Kopp et al. (2011), of 1.82-3.43 mg dL^{-1} Mg in rainbow trout farmed under intensive conditions.

Potassium plays roles in nerve function, muscle contraction, acid-base balance, and osmoregulation in rainbow trout. The values obtained in this study are situated between the values obtained by Holk & Lykkeboe (1998), namely 2.2 mmol L^{-1} in resting rainbow trout and 3.3 mmol L^{-1} when trout is exposed to higher waterflow.

Glucose levels in the plasma of rainbow trout did not differ significantly among groups ($p>0.05$; one-way ANOVA). G1 had the highest glucose level (129.98±70.01 mg dL^{-1}), followed by G2, G4, Gi, and G3, with 113.18±21.81, 109.1±51.07, 102.44±48.59 and 68.78±11.29 mg dL^{-1}, respectively. GLC is a critical energy source in rainbow trout plasma, supporting metabolism, stress response, and overall physiological function. Choi & Weber (2015)

note a blood glucose level of 225.2 mg dL^{-1}, in hyperglycemic rainbow trout. Normal glucose levels are around 108 mg dL^{-1} (Weber & Shanghavi, 2000; Polakof et al., 2012).

TB decreased by the end of the experiment, from 0.21±0.41 mg dL^{-1} in Gi, to 0.078±0.14, 0.006±0.008, 0.006±0.008 and 0.008± 0.011 mg dL^{-1} in G1, G2, G3 and G4, respectively. TB is low in the plasma of rainbow trout. TB in rainbow trout is an indicator of liver function, Ery breakdown, and overall metabolic health. The values obtained in our study are in line with values obtained by other authors, namely under 0.12 mg dL^{-1} (Sakai & Kawazu, 1978), and under 0.09 mg dL^{-1} (Manera & Britti, 2006). However, the changes were not statistically significant ($p>0.05$; Kruskal-Wallis test).

CONCLUSIONS

The hematological profile and plasma biochemistry of rainbow trout fed throughout the winter with diets supplemented with phytoadditives showed generally normal values for low water temperatures.

Notable differences in the hematological profile were observable in MCV, which increased in rainbow trout fed a 2% carrot meal supplemented feed and in the rainbow trout fed a 2% spinach meal feed, and MCHC, which decreased in all groups. CRN levels decreased in all groups, with a significant decrease in the conventionally fed rainbow trout.

CHOL and TG levels increased in all groups, possibly due to slowed metabolism and gonadal maturation.

The enzymatic profile did not suffer significant changes.

Some of the indices of the mineral profile suffered changes. Ca was highest in rainbow trout fed a 2% carrot meal supplemented feed, Na was highest in rainbow trout fed a 2% tomato meal supplemented feed, and K was highest in rainbow trout fed a 2% spinach meal supplemented feed.

This could be attributed to the higher mineral content of the phytoadditives, in corroboration with the slowed metabolism.

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NON-BREEDING RANGE IN SAHEL OF LESSER KESTRELS ORIGINATING FROM RECOVERED BULGARIAN POPULATION

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Abstract

The Lesser Kestrel (Falco naumanni) is a long-distance migratory species, with its primary breeding areas in Spain, Italy, and Greece, and smaller populations in Portugal, France, Bulgaria, and other countries. Birds from the three peninsulas (Iberian, Apennine, and Balkan) follow distinct migratory routes to reach their non-breeding areas in the Sahel. This study aims to identify and describe the core wintering areas of the Bulgarian population, focusing on migratory patterns and habitat use during the non-breeding season. We hypothesized that the spatial distribution and individual presence in these areas would reveal patterns of site fidelity and migration strategies. The study was based on satellite tracking data from Lesser Kestrels tagged in Bulgaria, with data from nine individuals tracked over 11 winters. Two core wintering areas were identified: the first, covering about 138,700 km², spans territories in Niger and Nigeria; and the second, covering over 78,458 km², is located in central and southeastern Chad. These two areas are critical zones, showing relatively high concentrations of individuals, indicating their importance for roosting and foraging, and emphasizing the need to protect these habitats.

Key words*: Balkan population, Falco naumanni, wintering grounds.*

INTRODUCTION

The Lesser Kestrel (*Falco naumanni*) is a migratory raptor that exhibits specific wintering patterns, migrating from breeding grounds in southern Europe to wintering areas in the Sahel region of Africa (Iñigo & Barov, 2010). After the end of the breeding season, the birds initially disperse and later begin to migrate on a broad front (Forsman, 1999). Understanding this species' seasonal movements and wintering behavior is crucial for effective conservation strategies (Briedis & Bauer, 2018; Kramer et al., 2018), as these periods and sites are key to its survival. Despite the importance of the wintering phase, detailed information on the locations and characteristics of these areas remains scarce, especially concerning the species' interaction with its environment during the non-breeding period.

Migratory birds divide their annual cycle between two areas separated by hundreds or thousands of kilometres (breeding and non-breeding grounds), usually under different environmental conditions and selective pressures, such as food distribution and abundance, offspring rearing constraints, and competition (Briedis & Bauer, 2018; Kramer et al., 2018). The movements of migratory birds are driven by a complex set of environmental factors that often differ between breeding and non-breeding areas. In Lesser Kestrel's case, identifying key wintering areas is particularly important for understanding how environmental factors influence their survival during the non-breeding period.

In recent years, advancements in tracking technology have allowed researchers to gather more precise data on the ecology, evolution and behaviour of migratory birds (López-López, 2016; López-Ricaurte et al., 2023; López-Ricaurte et al., 2024).

Based on the satellite tracking dataset from nine individuals of the Bulgarian population tracked over 11 winters, we performed a spatial and temporal analysis of wintering areas use and migratory phenology in Lesser Kestrel.

This study aims to identify and characterize core wintering areas of Lesser Kestrel in Niger, Nigeria, and Chad (Sarà et al., 2019), focusing on migratory patterns and habitat use during the non-breeding season. We hypothesized that the spatial distribution and individual presence within these areas would reveal patterns of site fidelity and migratory strategies. Additionally, we expected the wintering duration to be relatively consistent among individuals, with entry and exit dates clustering around similar timeframes. Areas with high concentrations of individuals were anticipated to emerge as critical conservation priorities (Iñigo & Barov, 2010), guiding future monitoring and protection efforts to ensure the species' survival.

MATERIALS AND METHODS

Study Area and Period

In the past, the species was widely distributed and numerous in Bulgaria, but by the end of the 20th century its numbers decreased significantly. In the early 21st century, there were no confirmed data of breeding in the country. In 2014, the Lesser Kestrel was restored successfully as a breeding species in Bulgaria by Green Balkans NGO in collaboration with DEMA and EuroNatur, on the territory of "Sakar" SPA part of NATURA 2000. Later, another unknown breeding site was found in the Burgas region (Gradev et al, 2016a; Marin et al, 2020). In 2021, there are four different breeding territories in Bulgaria - located from 8 to 243 m above sea level and the national population is estimated to be more than 40 pairs (Gradev et al., 2016a; Gradev et al., 2021). We tracked the movement of birds between their breeding territories in Bulgaria and their main wintering grounds in Africa, with our attention focused on the wintering areas in the Sahel, specifically in Niger, Chad, and Nigeria (Sarà et al., 2019), which are the main wintering locations for the Balkan population, particularly the Bulgarian subset, during the non-breeding period. The research period spans from 2015 to 2024.

Data Collection

The study was conducted using different types of satellite transmitters' GPS data from Lesser Kestrels tagged in Bulgaria and originating from the recovered population of the species in the country (Gradev et al., 2016b). Between 2014 and 2024, 9 satellite transmitters (5g Solar PTT-100 backpack - Platform Terminal Transmitters, Microwave Telemetry – 7 pieces), and GPS/GSM transmitters (one GPS-GSM Logger MINI, provided by INTERREX-RINGS) and one Wildlife Tracker HQBG1204 Hunan Global Messenger Technology Co., Ltd. were used (Gradev et al., 2016c). The first two types of devices were attached to the back of the birds using the standard "backpack" method (applied to eight individuals) shown on Figure 1 (Garcelón, 1985) and the last type was attached using the leg-loop harness method (Mallory & Gilbert, 2008). The combined weight of the harness (transmitter, teflon ribbons, stitching, and glue) used to secure the device to the bird's body did not exceed 2.4-2.8% of the bird's body mass. Following the guideline that the total weight of the equipment should not surpass 4% of the bird's weight, this ensures that the behaviour of the birds of prey is not negatively impacted (Sergio et al., 2015).



Figure 1. A real-size model of male Lesser Kestrel with a satellite transmitter attached on the back of the bird as a "backpack" (original)

Based on the tracking dataset of Bulgarian individuals (n = 9; 8 females and 1 male), we performed a spatial and temporal analysis of wintering site use and migratory phenology of the Lesser Kestrel.
The dataset consists of locations recorded in the Sahel region during the non-breeding period, focusing on the central-eastern Sahel.

Spatial Density Analysis
To identify and characterize the wintering areas, a spatial density analysis was performed using the Density Analysis Plugin in QGIS, which implements the Kernel Density Estimation (KDE) technique. This method assigns a density value to each pixel based on the proximity of points within a specified radius, allowing for the identification of areas with high concentrations of individuals. The technique helped identify not only the general density of individuals but also the specific zones that truly concentrate their activity during the non-breeding period.
For the density calculation, a pixel size of 250 meters and a search radius of 500 meters were selected to assess the overall spatial distribution of activities across the study area. The resulting continuous raster map represented the relative density of the locations, highlighting areas with higher concentrations of individuals during the non-breeding season.
The contour lines were then transformed into vector polygons representing high-density areas. These polygons were merged into two distinct areas, named Polygon 1 and Polygon 2, which encompass the main concentration zones of Lesser Kestrels during the wintering period.
A total of 450 GPS positions were identified within Polygon 1 and 327 GPS records within Polygon 2, providing a detailed representation of the spatial distribution of the individuals.

Statistical Analysis
A statistical analysis was performed using R to assess the migration patterns of Lesser Kestrels during the wintering period. The analysis focused on defining the arrival date as the first GPS record within each polygon and the exit date as the last GPS record. A new season was defined when a gap of 80 days between records occurred. The number of days each individual stayed within each polygon was calculated by determining the time between the arrival and exit dates. To summarize the temporal data, we calculated the arithmetic mean (± SD) and 95% confidence intervals for both the duration of stay and the entry and exit dates for individuals within each zone. These statistical measures helped quantify the variability in the migration timing and duration, providing insights into the wintering habitat use and migratory behaviour of the species.

RESULTS AND DISCUSSIONS

Wintering Areas and Phenology of Lesser Kestrel
The spatial density analysis using Kernel Density Estimation (KDE) in QGIS identified two core wintering areas for the Lesser Kestrel in the Sahel. The first polygon, covering approximately 138,700 km², spans regions in Niger and Nigeria, with a higher concentration in Niger. The second polygon, covering 78,458 km², is located in central and south-eastern Chad. The distance between the breeding and wintering areas is nearly 3,500 km, while the distance between the two polygons is approximately 800 km.
Figure 2 illustrates the spatial distribution of individuals within these polygons, highlighting their concentration during the wintering period. It provides a clear visual representation of both the identified wintering areas and the locations of the individuals within them.

Individual Presence and Variability
In this study, individuals located within the identified wintering areas were considered. In Polygon 1, a total of four individuals were included, although one of them only had the entry date available, as it disappeared abruptly without records of departure or duration. In Polygon 2, five individuals were considered, with a similar situation in one of them, which only had the entry date recorded due to its sudden disappearance.
Individuals 5N and B00 used both polygons but in different wintering seasons. No individual was recorded using both areas within the same season, indicating a pattern of site fidelity within a given year but flexibility across different years.

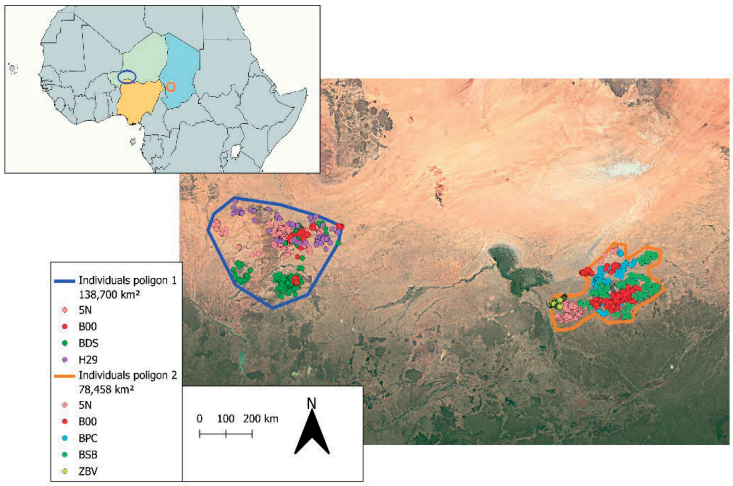


Figure 2. Spatial distribution of Lesser Kestrel individuals within the identified wintering areas in the Sahel, based on Kernel Density Estimation (KDE) analysis (original)

Timing of Migration and Duration of Stay

The wintering period, marked by the time spent within these polygons, varies across individuals. In Polygon 1, the mean entry date was September 30 ± 5.03 days (95% CI: September 24 to October 6, n = 4). In Polygon 2, the mean entry date was October 6 ± 7.59 days (95% CI: September 24 to October 18, n = 5). These results indicate that individuals in Polygon 2 tend to arrive later and exhibit greater variability in entry dates compared to those in Polygon 1.

Exit dates show only slight differences. In Polygon 1, the mean exit date was March 16 ± 14.48 days (95% CI: February 27 to April 3, n = 3), whereas in Polygon 2, the mean exit date was March 12 ± 18.43 days (95% CI: February 12 to April 10, n = 4). This suggests that individuals in Polygon 2 tend to depart slightly earlier, with some variability in their exit dates.

The mean duration of stay in Polygon 1 was 168 days ± 16.14 days (95% CI: 148-188 days, n = 3), while in Polygon 2, it was 158.5 days ± 15.41 days (95% CI: 132.96–184.04 days, n = 4). Therefore, individuals in Polygon 1 remained an average of 10 days longer than those in Polygon 2, though the variability in duration is similar between both areas.

The two core wintering areas for the Lesser Kestrel identified in this study confirmed the existing until now presumption for the wintering areas of the Balkan population of the species. Until now, the non-breeding areas in Africa for Mediterranean birds from the central-eastern regions (including Italy and the Balkans) were unidentified, as there was a lack of data on sub-Saharan ring recoveries or migration tracking (Sarà et al., 2019).

The timing of migration and the duration of stay within the wintering polygons show consistent patterns by the Lesser Kestrels. The entry dates for both polygons are similar, although individuals in Polygon 2 tend to arrive slightly later, with greater variability. The data from the satellite transmitters showed that the birds did not follow the same routes as other species along the coast until reaching Africa, through Via Pontica; they made direct flights to Africa across the Mediterranean Sea, and entered through Libya and Egypt (Gradev, 2018; Sahili, 2022). However, these differences are not substantial, indicating relatively synchronized migration.

Exit dates showed minimal differences, with individuals in Polygon 2 departing slightly earlier. While synchronization remained high, these small variations could be attributed to differences in resource availability or environmental conditions. This aligns with the idea that migration is driven by seasonal variations in food availability, climatic conditions, and other ecological factors, leading to coordinated movement of individuals within

a relatively short time window (Cote et al., 2017).
The duration of stay is slightly longer in Polygon 1, but variability is similar in both polygons. This suggests that, although some individuals stay longer in one polygon, habitat conditions are comparable in both areas.

CONCLUSIONS

The identification of the core wintering areas of the Lesser Kestrel has provided crucial insights into its migratory and non-breeding behaviour. The two polygons identified in Niger, Nigeria, and Chad represent critical zones where individuals concentrate their activity during the non-breeding period, highlighting the need to preserve these habitats. Both polygons show relatively high concentrations of individuals, suggesting that these regions serve as key roosting and foraging grounds for the species.
The results also confirm that the spatial distribution and presence of individuals in these areas reflect patterns of site fidelity. The entry and exit dates were relatively consistent among individuals, indicating a synchronization in the start and end of the wintering period. This underscores the importance of maintaining the quality of these habitats and protecting them from potential anthropogenic disturbances.
Given the essential role these areas play in the species' lifecycle, it is crucial to implement targeted conservation measures to safeguard them from future threats (Iñigo & Barov, 2010).

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EFFECTS OF CLIMATE CHANGE ON *Cyprinus carpio* (Linnaeus, 1758) IN THE DANUBE RIVER (2021-2024): ANALYSIS OF SEX RATIOS, CONDITION AND LENGTH-CLASS DISTRIBUTION

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Abstract

The common carp Cyprinus carpio (Linnaeus, 1758) is a key species in the Danube River, both ecologically and economically. The carp population in the Danube is affected by a range of factors such as overfishing, loss of habitats, pollution, anthropogenic disturbance and alien species. Climate change is putting an additional strain on these fish populations, altering parameters such as length and sex structure, Fulton's condition factor. This study examines changes in these parameters within carp populations in the Danube based on data collected from 2021 to 2024. By comparing biometric and demographic indicators, this research identifies hydrological trends driven by climatic variations, and how the sex structure was also affected, with sex ratio depending on region and local climatic conditions. Fulton's condition factor was calculated to assess the nutritional status and overall health of individuals in the population. This study highlights the urgent need for sustainable fisheries management and appropriate conservation strategies to address the challenges posed by climate change.

Key words*: Danube River, fisheries management, Fulton's condition factor, global warming, wild fish.*

INTRODUCTION

The Danube River, the second-longest river in Europe, represents a vital ecosystem, supporting rich aquatic biodiversity and providing numerous economic benefits (Ibanescu et al., 2016). Among the fish fauna species, the common carp *Cyprinus carpio* (Linnaeus, 1758) stands out due to its ecological and economic importance. I

t is one of the most widely distributed freshwater fish species globally (Cocan & Miresan, 2018; Anin et al., 2021; Dobrota et al., 2024), playing an essential role in maintaining aquatic biodiversity and supporting fisheries.

The common carp also serves as a key indicator of environmental health in the river (Weber & Brown, 2009).

However, the carp populations are affected by stress factors such as overfishing, habitat degradation, pollution, and increasingly high global temperatures.

In recent decades, climate change has become a critical threat to aquatic ecosystems (Dervis, 2007; Orr et al., 2024), influencing hydrological regimes, water temperatures, and habitat availability. These disturbances significantly impact the life cycles and population dynamics of native species (Seneviratne et al., 2021; Mariu et al., 2023). Due to its wide distribution and ecological relevance, the common carp is an ideal subject for analyzing the effects of climate change on fish population parameters.

The analysis of the sex ratio involves an unbiased sampling for studying the relative abundance of females and males within a

population, in our case, the common carp from the Danube River. This analysis provides valuable information about population structure and can be used to assess its health and dynamics.

The Fulton coefficient, also known as the condition factor, is a biometric indicator frequently used to evaluate the health status and overall condition of fish (Mozsar et al., 2014; Morado et al., 2017). It provides essential insights into the nutritional and physiological state of individuals, being particularly valuable for specialists in analyzing how carp cope with critical periods (Latiu et al., 2022; Savin et al., 2022; Thangaraji et al., 2023; Dobrota et al., 2024).

In the scientific literature, there is a significant gap in research addressing the impact of climate change on *C. carpio* populations in the Danube River, particularly concerning parameters such as sex ratio, Fulton condition factor, and size distribution. While general studies on fish fauna exist, detailed longitudinal and regional investigations are lacking to highlight the variability of these indicators under the effects of climatic pressures.

This study aims to fill this gap by providing a perspective on how climate change influences common carp populations in the Danube River. The research focuses on data collected between 2021 and 2024, offering a comprehensive view of *C. carpio* vulnerabilities. The findings emphasize the need to implement sustainable management strategies to mitigate the effects of climate change on freshwater ecosystems.

MATERIALS AND METHODS

Data collection. Was conducted by the research staff of Research and Development Institute for Aquatic Ecology Fishing and Aquaculture of Galati (I.C.D.E.A.P.A. *in Romanian*) during scientific fishing sessions held in all four seasons, between 2021 and 2024 along the entire Romanian section of the Danube River, from Moldova Nouă to Galați (Figure 1).

Fishing was carried out using two types of gear, fixed and drift gillnets. The dimensions of the fishing gears were as follows: for fixed nets, the set length ranged between 100 m and 200 m, the vertical height varied between 2.5 m and 3.5 m, and the mesh size (a) measured between 40 mm and 60 mm (knot to knot). For drift nets, the set length ranged between 150 m and 200 m, the vertical height could vary between 2.5 m and 4.0 m, while the mesh size (a) ranged between 40 mm and 80 mm (knot to knot).

According to Order 342/2008, the legally allowed minimum size for common carp is 40 cm, which justifies the dimensions of the fishing gear used.



Figure 1. Danube River basin – Study area (Source: www.icpdr.ro)

For each common carp specimen, total length (cm), standard length (cm), and maximum height (cm) were recorded using an ichthyometer with ± 0.01 cm precision. Weight was measured using a scale with ± 0.01 g precision.

Environmental data for 2021-2024 were obtained from the National Institute of Hydrology and Water Management (https://www.hidro.ro/en/bulletin_type/diagnoza-si-prognoza-pentru-dunare/) for all hydrological stations located along the Danube River, and the annual mean values were calculated. The annual variation range was determined by identifying the minimum and maximum values for each year.

Data processing and graphs were performed using the XlStat program and R software version 4.4.2.

Analysis of Length-Class Distribution. The length-class distribution was analyzed to assess the demographic structure and health of the population. Length classes, set at 5 cm intervals (ranging from the minimum to the maximum lengths of captured specimens), were used to group individuals based on their size.

The frequencies for each class were calculated and data validation was performed using the Grubbs Test to identify outliers and the Shapiro-Wilk test to verify the normal distribution of the

frequencies per class each year. These validations are essential to ensure that the assumptions of subsequent statistical tests are met. The condition of homogeneous was confirmed through the Bartlett's Test (at a confidence level of 0.05). To evaluate differences between the years 2021-2024, a one-way ANOVA test was applied, comparing frequencies per year and identifying statistically significant differences.

Sex Ratio Analysis. The sex ratio was calculated annually, expressing the number of females relative to the number of males (e.g., 1:1, 2:1).
For statistical analysis, the data distribution was verified using the Shapiro-Wilks test at a 95% significance level, confirming normality for both sexes. According to Berk and Jones (1979) and Pearson (1900), the Chi-squared Goodness-of-Fit test was applied to compare the observed distribution with the expected proportions (Rashid, 2018).

Fulton's Condition Factor. According to Bagenal and Tesch (1978), Fulton's condition factor was calculated annually for each specimen using the formula: $K = 100*TW/TL^b$, where: K is Fulton's condition factor, TW represents total body weight (g), TL is total length (cm) and b represents a value demonstrating fish normal growth dimensions, from the weight-length relationship, expressed as: $TW = a*TL^b$, where intercept (a) describes the rate of change of weight with length and slope (b) gives information about the type of growth (Froese, 2006). The relationships between the weight and the length of fish were determined through linear regression (Log TW = Log a + b*Log TL) (Stavrescu- Beldivan et al., 2022).
A value of b greater than 3 indicate a positive allometric growth pattern, while when b is less than 3 shows a negative or hypoallometric growth pattern (Karachle and Stergiou, 2012).
This condition index is used to compare fish populations from different habitats or to monitor seasonal changes in their condition. A K>1 indicates good health and nutrition, while a K<1 suggests poor condition, possibly due to environmental issues or limited access to food (Nash et al., 2006; Vishwakarma et al., 2024).
Fulton's condition factor was calculated separately for males and females. This index varies significantly depending on species, habitat, and season, emphasizing the need for contextualized interpretation (Ricker, 1975; Blackwell et al., 2000). Fulton's condition factor is essential for monitoring fish populations in natural environments and aquaculture, providing valuable insights into environmental quality and the trophic dynamics of ecosystems.
Statistical analysis for Fulton's Condition Factor for the analyzed period included the Kruskal-Wallis Test, due to non-normal distribution for both genders. The Kruskal-Wallis test is a non-parametric test used to compare the medians of two or more independent groups. The test was chosen to assess whether there are significant differences between distributions of the condition values across annual data (Siegel, 1988; Corder & Foreman, 2009). Post-hoc analysis was conducted using Dunn's test to precisely identify which groups are significantly different and Bonferroni correction was applied to minimize Type I errors (Dunn, 1964; Costa et al., 2015).

RESULTS AND DISCUSSIONS

Between 2021 and 2024, a total of 1956 carp specimens were captured, with a combined weight of 6315.59 kg. The morphological parameters of the catches, broken down by year, are presented in Table 1 (for mean values, standard deviation is provided).

Analysis of Length-Class Distribution

The distribution of individuals across length classes provides information about the demographic structure, the health of the carp population, and environmental conditions, including the impact of climate change, food availability, and spawning habitats (Stavrescu-Bedivan et al., 2015). The presence of multiple length classes indicates a diverse population with good recruitment and survival rates (Jin et al., 2015). The dominance of a single length class may indicate overfishing, increased mortality, or unsuitable environmental conditions, while the absence of certain classes reflects issues with recruitment or survival.

Table 1. Morphometric parameters of the common carp specimens captured during the 2021-2024 period (No. ex.: number of fish sampled; mean ± standard deviation, min., max.).

Specification	No. ex.	TW (kg)	W mean (kg)	W min. (kg)	W max. (kg)	TL mean (cm)	TL min. (cm)	TL max. (cm)
2021	467	2200.20	4.71±2.19	0.80	8.30	66.88±12.55	41.00	90.00
2022	453	1456.33	3.21±1.21	1.00	7.00	56.44±8.14	30.00	80.00
2023	516	1323.33	2.56±1.03	0.50	5.70	54.85±6.99	30.00	71.00
2024	520	1335.73	2.57±1.02	0.50	5.70	54.88±6.98	30.00	71.00

NB. No. ex. - number of fish sampled; mean ± standard deviation; minimum value, maximum value.

Bartlett's Test show homogenous variances (for df=3 and α=0.05 resulted $\widehat{\chi^2}$=7.18<χ^2_{critic}=7.81) for the frequencies in each class between 2021 and 2024, while ANOVA results (for df=3, 48 and α=0.05 $\hat{F}$= 0.057<Fcritic = 2.79) showed no differences between the group means (i.e., the frequencies per class per years 2021-2024). This suggests that, although differences in means and standard deviations were observed, they were not large enough to be statistically significant. However, the observation of the distribution curves (Figure 2) indicates a slightly different patterns in length distribution from 2021 to 2024.

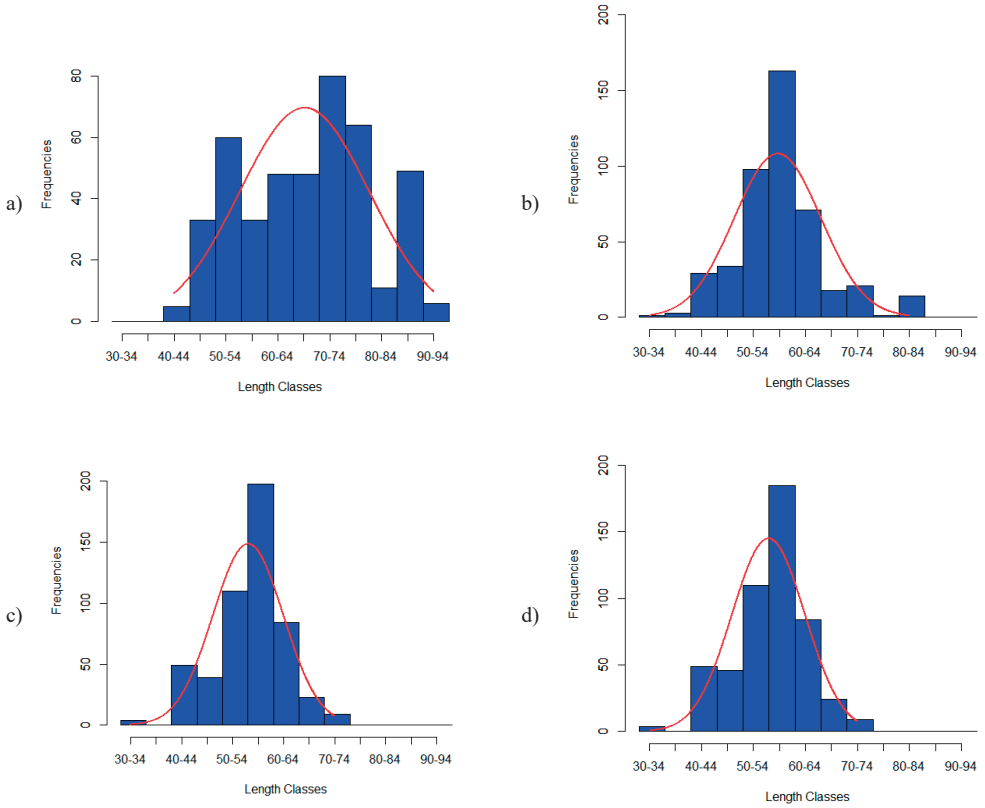


Figure 2. Total Length distribution of common carp population in the Danube River, 2021-2024 period (in cm): a) 2021; b) 2022; c) 2023; d) 2024.

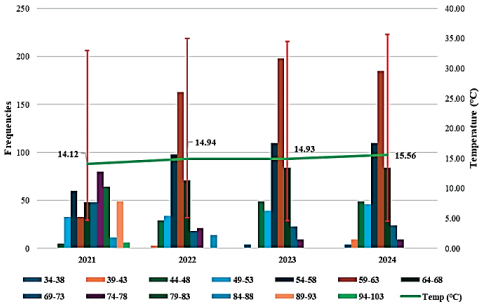


Figure 3. Total Length class distribution and mean annual water temperature

The range of the length distribution recorded in 2021 shows 4 length classes beyond the 70-74 class, we observe 2 in 2022 but none in 2023 and 2024.

Between 2022 and 2024, a significant increase in the number of individuals in the 59-63 cm length class was observed as water temperatures rose, from 33 ind. in 2022 to 163 ind. in 2023, peaking at 198 ind. in 2023 before slightly decreasing to 185 ind. in 2024 (Figure 3).

In 2023, a higher number of specimens was also recorded in the 44-59 cm length classes. However, in 2024, the numbers for the 39 and 44 cm classes remained stable, while those for the larger classes (79, 84, and 89 cm) decreased. Similar trends were reported for the Danube Delta by Stroe et al. (2022).

Overall, there has been a gradual decrease in the number of individuals in the larger length classes, alongside a reduction in the average length of the population, from 66.28 cm in 2021 to 54.40 cm in 2024. Additionally, CV has decreased from a maximum of 18.47% in 2021 and a minimum of 12.74% in 2023, indicating lower variability in the size of individuals, while the slight increase in 2024 to 13.44 may indicate either a diversification of lengths (e.g., the recruitment of younger individuals or the survival of larger ones) or a change in population structure.

Sex Ratio Analysis

The sex ratio (F/M) for the period 2021-2024 indicates a predominance of males. This observation may reflect ecological or biological factors that favour male survival. The results of the Chi-square test (Table 2) show significant deviations from the theoretical balance ratio in the years 2021, 2022, and 2024 ($\hat{X}$-squared>$X_{critical}$). In 2023, the ratio does not differ significantly from equilibrium ($\hat{X}$-squared < $X_{critical}$).

Table 2. Chi-square Test: Comparison of Annual Values

Specification	2021	2022	2023	2024
Chi-squared	9.047	6.20	3.10	8.93
Critical Chi-squared	3.84	3.84	3.84	3.84
p-value	0.030	0.145	0.481	0.066

The evolution of the F/M ratio (Figure 4) shows a trend towards equilibrium in 2023, followed by a slight decrease in 2024, although that does not necessarily indicate a critical population imbalance. Deviations from the 1:1 ratio do not inherently signal a problem but rather reflect natural fluctuations influenced by environmental conditions, fishing pressure, and biological dynamics. In the case of common carp, such variations are common and may be linked to recruitment success, habitat changes, or selective fishing practices, rather than an immediate threat to population stability.

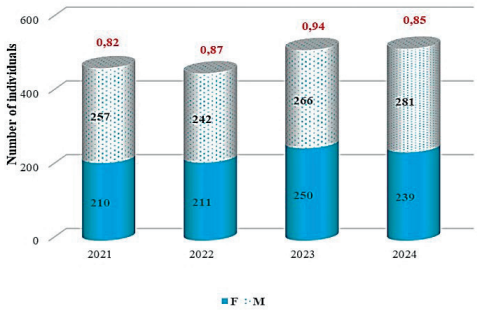


Figure 4. Sex Ratio in common carp population between 2021 and 2024

Previous research has highlighted a masculinization effect in the species, under the effect of elevated temperatures (Biswas et al., 2021), a trend that is also reflected in the F/M ratios obtained in this study.

The values are much higher compared to those recorded in 1996 (Ciolac, 2004) - ranging from 0.42 to 0.65, which suggests either an increase in the proportion of females in the population or a decrease in the number of males.

In the analysis of the relationship between water level and water temperature with the sex ratio, moderate influences of both variables on the sex

distribution in the population are observed. A higher water level, as seen in 2023 (437.27 cm), is associated with an increase in the sex ratio, which may indicate a positive effect of favourable environmental conditions on the proportion of males to females. At the same time, water temperature, which ranged between 14.12-15.56°C. Also influences this distribution, impacting fish development and reproduction. Although the relationship is weak (Pearson coefficient = 0.4 between Sex Ratio and water level, respectively, 0.3 for water temperature), these environmental conditions may play a significant role in determining sex ratios, influencing reproductive success and the overall development of the population (Geffroy & Wedekind, 2020).

Fulton's Condition Factor

The Kruskal-Wallis test result revealed significant differences across the annual values of K for both females ($p<0.05$ and $\chi^2 = 741.59 > \chi^2_critical = 7.81$, with $df = 3$ and $\alpha = 0.05$) and males ($p<0.05$ and $\chi^2 = 900.4 > \chi^2_critical = 7.81$, with $df = 3$ and $\alpha = 0.05$) (Figure 5a). The Dunn test for the female individuals, adjusted with the Bonferroni correction, showed pronounced contrasts between 2022-2024 ($Z = 24.51$, $p.adj \approx 7.71 \times 10^{-132}$) and 2021-2024 ($Z = 20.11$, $p.adj \approx 3.85 \times 10^{-89}$).

A similar pattern was observed also for males, where the Dunn test, also adjusted using the Bonferroni correction, confirmed significant differences across all years. The highest Z value was recorded for the 2021-2024 comparison ($Z = 27.59$, $p.adj \approx 9.99^{-167}$), although all other comparisons also yielded equally significant results (Figure 5b).

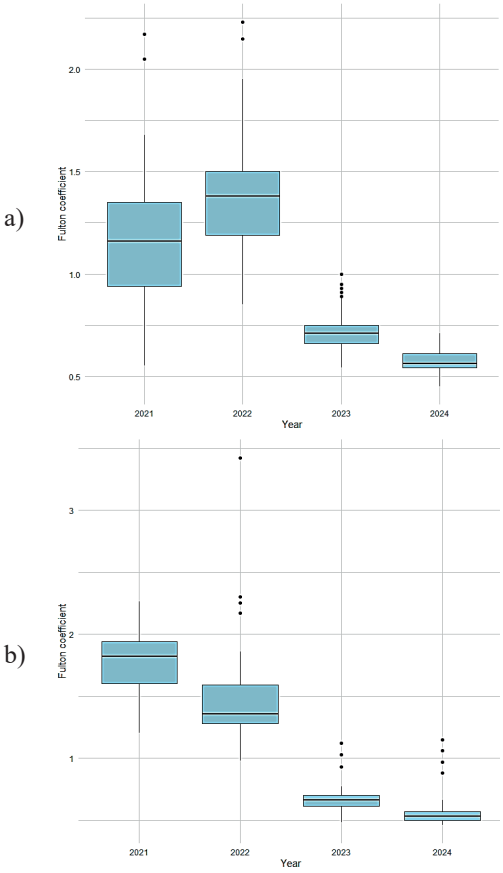


Figure 5. Fulton's Condition Factor.
Annual values distribution between 2021 and 2024:
a) Females; b) Males

Table 3. Fulton's Condition Factor

Specification	Females					Males				
Years	Kmin.	Kmax.	Kmean	a	b	Kmin.	Kmax.	Kmean	a	b
2021	0.54	2.17	1.16±0.25	0.0113	3.0524	1.2	2.26	1.78±0.22	0.0177	2.9430
2022	0.85	2.23	1.36±0.23	0.0135	3.0324	0.98	3.42	1.42±0.24	0.0141	3.0234
2023	0.43	0.80	0.56±0.06	0.0056	3.2475	0.48	1.12	0.65±0.08	0.0054	3.2011
2024	0.44	0.71	0.58±0.05	0.053	3.2532	0.46	1.47	0.54±0.07	0.0054	3.2181

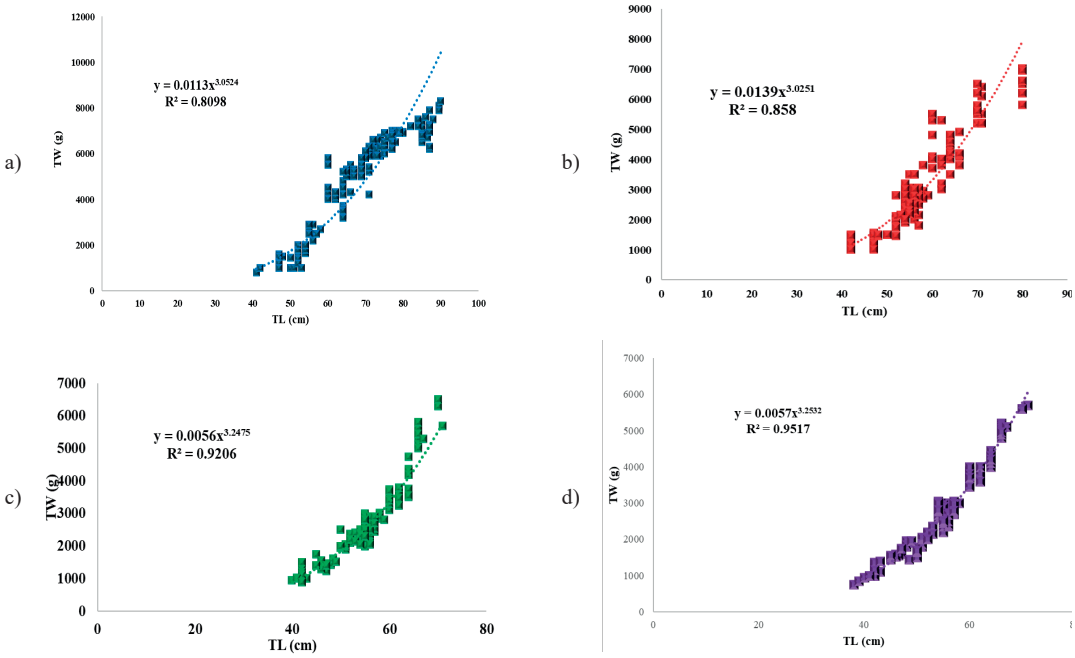


Figure 6. Weight-Length Relationship for carp female: a) 2021; b) 2022; c) 2023; d) 2024

For female, the annual WLRs were expressed as:

Log (TW) = 10864* log (TL) – 40791; TW = 0.0113* $TL^{3.0524}$ with r^2 =0.81 (confidence level of 95% for the intercept and slope) - 2021 (Figure 6a).

Log (TW) = 9527.1* log (TL) – 35359; TW = 0.0135* $TL^{3.0324}$ with r^2 =0.86 (confidence level of 95% for the intercept and slope) - 2022 (Figure 6b).

Log (TW) = 8003* log (TL) – 29360; TW = 0.056* $TL^{3.2475}$ with r^2 =0.92 (confidence level of 95% for the intercept and slope) - 2023 (Figure 6c).

Log (TW) = 7826.3*log (TL) - 28540; TW = 0.0057* $TL^{3.2532}$ with r^2 =0.87 (confidence level of 95% for the intercept and slope) - 2024 (Figure 6d).

Growth type for female carp caught between 2021 and 2024 showed a positive allometric pattern, with b > 3 for each year, suggesting that the fish biomass increases faster than length, so the organism becomes more robust or stocky, while the strong r^2 shows a strong correlation between biomass and length.

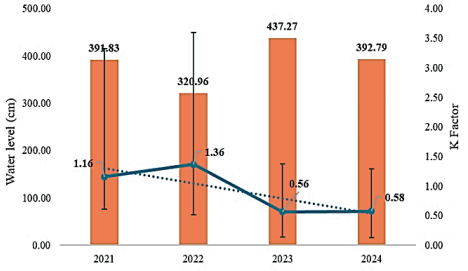


Figure 7. Evolution of K factor (female values) in correlation with Danube's annual mean water level between 2021 and 2024

The annual average values of female K for 2021-2024 range between 0.56 in 2023 and 1.36 in 2022. The variations are shown in Table 3 and Figure 7, suggesting that the Fulton factor was good in 2021-2022, but dropped sharply in 2023, with no clear signs of recovery in 2024. Combined with the decrease in the error bars, this trend indicates a general and uniform deterioration in fish condition, not only for a few individuals, but for the whole population.

This indicates a possible deterioration of environmental conditions or other negative ecological changes.

A Pearson coefficient of -0.82 between K and water level suggests a strong negative correlation, meaning that as the water level increases, the Fulton's condition factor (K) tends to decrease. This implies that higher water levels may negatively impact the overall condition of the fish, possibly due to factors such as changes in food availability, habitat alteration, or increased energy expenditure required for movement and foraging.

For male individuals, the annual WLRs were expressed as:
Log (TW) = 10556* log (TL) – 39625; TW = 0.0177* $TL^{2.943}$ with r^2 =0.94 (confidence level of 95% for the intercept and slope) - 2021 (Figure 8a).
Log (TW) = 8517.5* log (TL) – 31271; TW = 0.0141* $TL^{3.0234}$ with r^2 =0.92 (confidence level of 95% for the intercept and slope) - 2022 (Figure 8b).
Log (TW) = 6683.6* log (TL) – 24118; TW = 0.0054* $TL^{3.2011}$ with r^2 =0.96 (confidence level of 95% for the intercept and slope) - 2023 (Figure 8c).
Log (TW) = 6577.1*log (TL) - 23964; TW = 0.0054* $TL^{3.2181}$ with r^2 =0.97 (confidence level of 95% for the intercept and slope) - 2024 (Figure 8d).

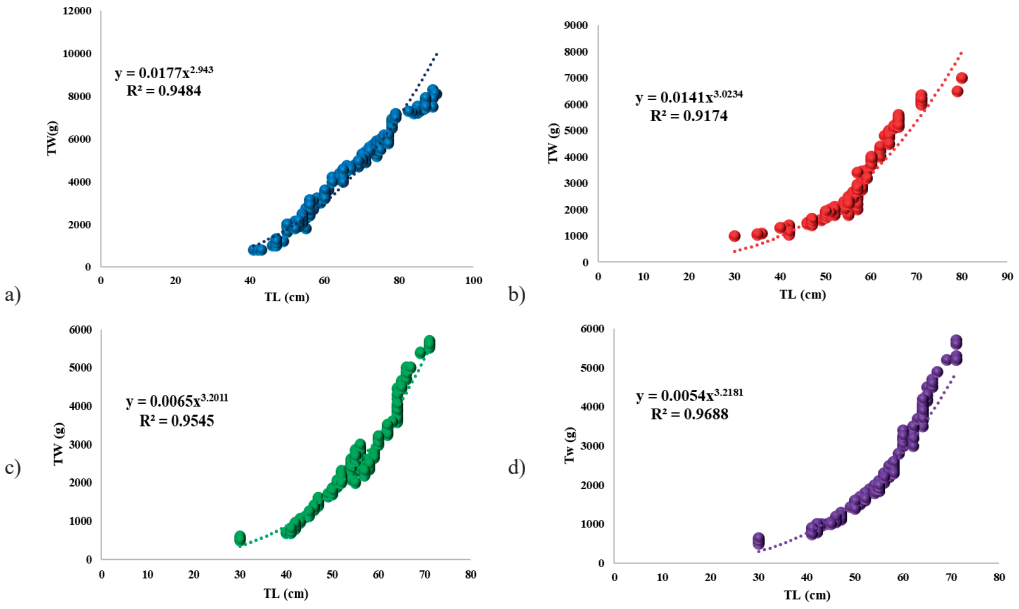


Figure 8. Weight-Length Relationship for carp male: a) 2021; b) 2022; c) 2023; d) 2024

Between 2021 and 2024, the growth type of male carp varied from a hypoallometric type in 2021 (with b < 3), suggesting that growth in length was proportionally greater than weight gain, to a positive allometric growth from 2022 on, indicating improved conditions and a more balanced energy allocation towards body mass accumulation.

The annual average value of male K ranged from 0.54 in 2024 to 1.78 in 2021 (Table 3, Figure 9), underlining the same decline as in female carp population, which suggests a consistent downward trend in body condition over the years.

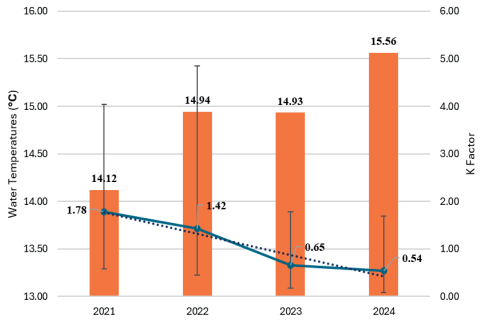


Figure 9. Evolution of K factor (male values) in correlation with Danube's water annual mean temperatures between 2021 and 2024

With a strong negative correlation of -0.85, the male K factor is influenced by water temperature, meaning that as the water temperature increases, the Fulton's condition factor (K) tends to decrease. Chezik et al. (2014) utilized maximum and minimum air temperature values, or the number of days above a certain threshold, to demonstrate the impact of climate change on aquatic ecosystems. This study explores the annual variation in water temperature between 2021 and 2024 and examines how these fluctuations influence the variation in the Fulton's condition factor (K), providing further insights into the relationship between climate dynamics and fish health in the Danube River.

A negative allometric growth in carp and a good condition of 1.98 (with no gender separation), was recorded also by Stavrescu-Beldivan et al. (2022) for the population Pond Bitina 3 in Ialomita County.

The differences between the values reported by Ciolac (2000) and the current ones could indicate an improvement in the condition of carp over the past decades. However, the downward trend observed in 2023-2024 and the negative correlation with water level and water temperature suggest a recent decline in fish condition, which could be linked to ecological or anthropogenic factors. As Lenhardt et al., 2012 and Milanov et al., 2016 sustained environmental factors, such as pollutants, especially heavy metals, have a significant negative impact on fish health and contribute to a decrease in Fulton's condition factor. The accumulation of these pollutants in the tissues of *C. carpio* can cause physiological stress, which is reflected in slowed growth and deteriorating physical condition (Milanov et al., 2016).

The improvements in 2022 and 2023 may reflect a positive adaptation to environmental conditions, physiological response to the shifting environment, but the decline in 2023-2024 could be a warning signal to be considered in the future, related to climate change, pollution, or other ecological pressures. The differences between the minimum and maximum values remain significant, highlighting the presence of individuals in both excellent and poor conditions.

Compared to other regions, the values obtained for *C. carpio* in the Danube are much lower than those reported by Zencir Tanir (2020) in Tercan Dam Lake, Eastern Anatolia, Turkey (2.148±0.030), and those reported by Daliri (2012) for the Caspian Sea (1.34±0.24). Also, in Lake Naivasha, Kenya in 2013-2014, the K factor was greater than 1 for both female and males as observed by Aera et al. (2014).

CONCLUSIONS

A gradual decrease in the average size of individuals was observed during the period 2021-2024, which may reflect both ecological pressures and the effects of climate change. The length class distribution indicates a reduction in the number of large individuals, signaling the possibility of overexploitation or other stress factors. The F/M ratio showed a tendency towards equilibrium during the analyzed period, increasing from 0.82 in 2021 to 0.94 in 2023, but with a slight decrease in 2024 (0.85). This fluctuating equilibrium could influence the reproductive success of the population.

The K factor was higher in 2021-2022 but experienced a sharp decline in 2023, with no clear signs of recovery in 2024, suggesting a general deterioration in the fish population's condition rather than affecting only isolated individuals. In females, K showed a strong negative correlation (-0.82) with water levels, indicating that higher water levels may negatively impact body condition, while in males, K exhibited an even stronger negative correlation (-0.85) with water temperature, suggesting that higher temperatures could adversely affect growth and overall condition. Regarding growth patterns, females consistently

maintained positive allometric growth (b>3) throughout the years, indicating a faster accumulation of body mass relative to length, whereas males transitioned from hypoallometric growth in 2021 (b<3, indicating greater length growth than weight gain) to positive allometric growth starting in 2022, reflecting a temporary improvement in conditions. However, the significant decline in the K factor in 2023-2024 may serve as a warning sign of the impact of climate change, pollution, or other ecological pressures on the aquatic ecosystem, contributing to stress on the common carp population, reflected in variations in population structure and individual condition. However, it is necessary to further analyze to what extent these factors have influenced the carp population.

The reductions in size and body condition in 2024 underline the urgency of adopting adaptive measures to conserve the *C. carpio* populations and protect the Danube ecosystem.

This study provides an integrated perspective on the impact of climate change on key parameters of the common carp population in the Danube River, highlighting the need for science-based management to ensure the resilience of this species.

ACKNOWLEDGEMENTS

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PARASITE COMMUNITIES OF FISH FROM THE MECHKA RIVER, MARITSA RIVER BASIN

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Abstract

This study aims to examine the component and infracommunity of the bleak, Alburnus alburnus (Linnaeus, 1758), from the freshwater ecosystem of the Mechka River, part of the Maritsa River basin, East Aegean region. For the study, 54 specimens of bleak were caught from the Mechka River - Parvomay biotope (Debar) in the autumn of 2024. The fish were examined for the presence of parasites using standard methods. Species from Trematoda, Acanthocephala, and Nematoda were identified. Representatives of Acanthocephala dominate. The following indices were examined: Brillouin's diversity index (HB), Pielou's evenness index (E), and Simpson's dominance index (C). The studied biotope is a new habitat for the identified bleak parasites.

Key words*: bleak, Bulgaria, East Aegean region, ecological indices, parasites.*

INTRODUCTION

The Mechka River falls into the Maritsa River basin, East Aegean region. The river rises from the foot of Samantepe Peak in the Western Rhodopes at 954 m above sea level, flows for 43 km and flows into the Upper Thracian Lowland on the right side of the Maritsa River in the area of the town of Parvomay at 117 m above sea level.
The Mechka River passes through the territory of two municipalities (Assenovgrad and Parvomay) and two settlements - the town of Parvomay and the village of Poroyna (Integrated Development Plan of Municipality of Parvomay, 2021-2027).
Fish and their parasites are a significant element of biodiversity in freshwater ecosystems (Kirin et al., 2020).
They are among the most used bioindicators for assessing the state of waters (Kuzmanova et al., 2023). Many anthropogenic activities lead to a deterioration in the quality of aquatic ecosystems (Biswal & Chatterjee, 2020; Gartsiyanova et al., 2022; Gartsiyanova et al., 2024). The species diversity of parasites decreases due to pollution and stress (Biswal & Chatterjee, 2020). The ecological indices of parasites are related to the conditions of the aquatic environment and reflect the presence of pollutants (Palm, 2011). There is usually an equilibrium in the parasite-host relationship. A large number of parasites infect fish and can lead to their death, but not all fish parasites are dangerous to human health. It is necessary to increase people's awareness of diseases caused by parasites (Tessema, 2020). Knowing about the parasite fauna of fish is important for protecting fish populations and human health. Currently, the parasite fauna of fish from the freshwater ecosystem of the river Mechka has not been studied.
This study aims to examine the component community and infracommunity of the bleak, *Alburnus alburnus* (Linnaeus, 1758), from the Mechka River, part of the Maritsa River basin, East Aegean region.

MATERIALS AND METHODS

In the autumn of 2024, 54 specimens of the bleak, *Alburnus alburnus* (Linnaeus, 1758), were caught from the Mechka River near Parvomay. The habitat is in the Debar district (42°04'27.2"N 25°11'48.4"E). It is located next to the bridge on the main road connecting the villages of Popovitsa and Byala Reka (Figure 1).

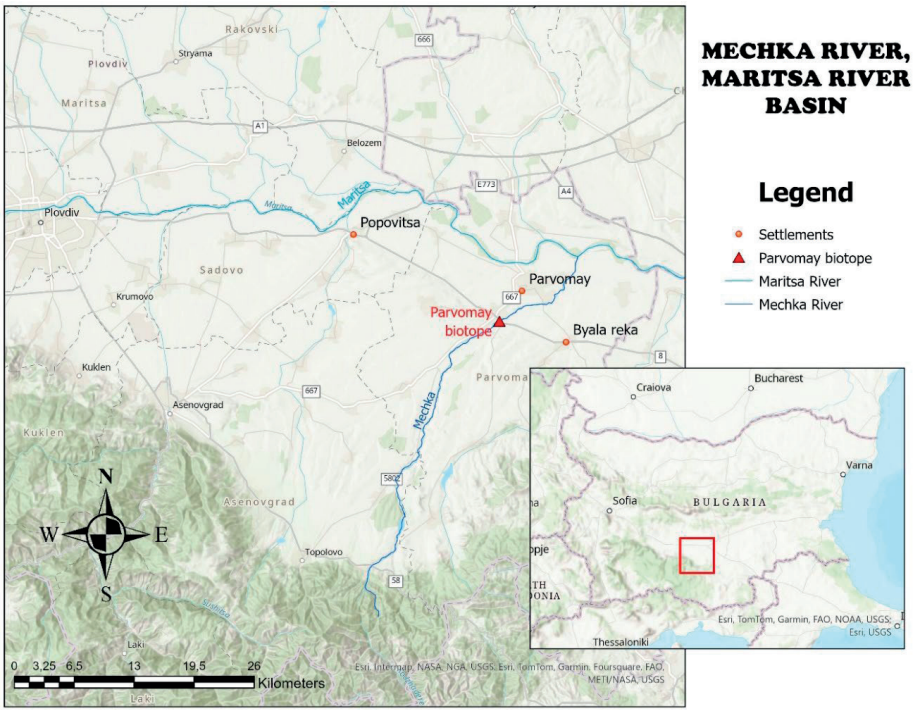


Figure 1. Map of the studied biotope of the Mechka River (original)

After issuing a ticket for amateur fishing, the fish were caught with a fishing rod. The caught fish species were determined using a handbook (Karapetkova & Zhivkov, 2006). After the fish were caught, a visual inspection of the body surface and abdominal cavity was performed, followed by a dissection of the organs. The parasitological examination of the fish was conducted in a laboratory at the Department of Chemistry, Phytopharmacy and Ecology, and Environmental Protection of the Agricultural University-Plovdiv. A Micros Austria MZ 1240 stereomicroscope and an XS-213 China microscope were used. Permanent microscopic preparations were prepared from the isolated trematodes using staining with carmine and passing through an ascending alcohol series according to Dubinina (Zaharieva, 2022). Temporary microscopic slides were prepared from the isolated acanthocephalans and nematodes using the glycerine-alcohol brightening method according to Zashev & Margaritov (Zaharieva, 2022); Moravec (2013). The parasite species were determined according to Bauer (Zaharieva, 2022) and Moravec (2013). The structure of the parasite communities was examined at two levels – component community, characterized by the ecological indices mean intensity (MI), mean abundance (MA) and prevalence (P%) according to Bush et al. (Zaharieva et al., 2025); and infracommunity represented by the indicators total number of species, mean number of species, total number of specimens, mean number of specimens, Brillouin's diversity index (HB), Pielou's evenness index (E) and Simpson's dominance index (C) according to Magurran (Zaharieva, 2022). According to the prevalence, parasite species are divided into core (P % > 20), component (10 < P % < 20) and accidental (P % < 10) by Kennedy (Zaharieva, 2022).

RESULTS AND DISCUSSIONS

Alburnus alburnus is a freshwater, brackish, benthopelagic fish. The species is native to almost Europe and Asia and was introduced to Cyprus, Italy, Portugal, Spain and Algeria. The bleak is a gregarious fish. It feeds on insects, invertebrates that have fallen to the water surface, and plankton (Froese & Pauly, 2025).

In the autumn of 2024, 54 specimens of bleak from the Mechka River, Parvomay biotope (Debar), were collected and subjected to parasitological examination. Infection with six parasite species was detected: two species of trematodes (*Posthodiplostomum cuticola* (von Nordmann, 1832) Dubois, 1936, localized in the skin, fins, gills; *Ichthyocotylurus pileatus* (Rudolphi, 1802) Odening, 1969, metacercariae, localized in the body cavity under the serous membrane of the intestine and the swim bladder); two species of acanthocephalans (*Acanthocephala anguillae* (Müller, 1780), with intestinal localization; *Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908, with intestinal localization) and two species of nematodes (*Pseudocapillaria tomentosa* (Dujardin, 1843) Lomakin & Trofimenko, 1982, with intestinal localization, mainly in the distal part); *Schulmanela petruschewskii* (Schulman, 1948) Ivashkin, 1964, with liver parenchyma localization).

In the component community of the bleak with the largest number of specimens, trematodes are represented (with > 803 specimens), followed by acanthocephalans (with 157 specimens) and nematodes (with seven specimens). Two core parasite species have been identified - *Ac. anguillae* (P % = 57.41) and *P. laevis* (P % = 46.30); one component and three accidental species. The highest values for MI and MA were found in the trematode *P. cuticola*, and the lowest in the nematode *Sch. petruschewskii* (Table 1).

Table 1. Component community of *Alburnus alburnus* from the Mechka River, Parvomay biotope (Debar) (N - number of fish studied; n - number of infected fish; p - number of parasites; MI - mean intensity; MA - mean abundance; P % - prevalence; R - range)

N = 54	N	p	MI	MA	P%	R
Posthodiplostomum cuticola (von Nordmann, 1832) Dubois, 1936, metacercariae	8	> 800	100.00	14.81	14.81	> 100
Ichthyocotylurus pileatus (Rudolphi, 1802) Odening, 1969, metacercariae	1	3	3.00	0.06	1.85	3
Acanthocephalus anguillae (Müller, 1780)	31	98	3.16	1.81	57.41	1-15
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	25	59	2.36	1.09	46.30	1-11
Pseudocapillaria (Pseudocapillaria) tomentosa (Dujardin, 1843) Lomakin & Trofimenko, 1982	5	6	1.20	0.11	9.26	1-2
Schulmanela petruschewskii (Schulman, 1948) Ivashkin, 1964	1	1	1.00	0.02	1.85	1

In the present parasitological study of 54 specimens of bleak, infection was found in 46 specimens (85.19%). More than 967 parasite specimens were studied. Twenty-four specimens of bleak were infected with 1 parasite species; 19 specimens - with 2 parasite species and 3 specimens of bleak - with 3 parasite species. The number of parasite specimens in one specimen of *Alb. alburnus* varies from 1 to more than 109. Pielou's evenness index (E) is low, which indicates that the conditions are not optimal. Simpson's dominance index (C) is high, which is associated with the dominance of one species – the trematode *P. cuticola* (Table 2).

A number of authors have studied the parasite fauna of bleak from the East Aegean region, including the catchments of the Maritsa, Arda, Tundzha, and Byala Rivers. Three parasite species found in *Alb. alburnus* from the Mechka River, Parvomay biotope (Debar) -*P. cuticola, Ps. tomentosa*, and *Sch. petruschewskii*, have not been reported in bleak from the East Aegean region (Table 3).

Table 2. Infracommunity of *Alburnus alburnus* from the Mechka River, Parvomay biotope (Debar)

Number of specimens *Alburnus alburnus*	**Number of parasites**			
	0	**1**	**2**	**3**
	8	24	19	3
Total number of species (Mean numbet of species ± SD)	6 (1.33 ± 0.82)			
Total number of specimens (Mean number of specimens ± SD)	> 967 (17.91 ± 36.19)			
Range	1- > 109			
Brillouin's biodiversity index (HB)	0.403			
Pielou's evenness index (E)	0.256			
Simpson's dominance index (C)	0.788			

Table 3. Parasites of *Alburnus alburnus* reported from the East Aegean region

Author	**River**	**Parasite species**
According to Margaritov (by Chunchukova et al., 2019)	Maritsa River, Chepinska River	*Caryophyllaeus brachycollis* (Janiszewska, 1951)
Kakacheva-Avramova, 1965	Maritsa River	*Caryophyllaeidae* gen sp.; *Rhabdochona* sp. juv.
	Maritsa River (Pazardzik) and some canals; Topolnitsa River and canals; Chepinska River; Asenitsa River; Chetiridesette izvora (Asenovgrad Region); Sushitsa River and canals; Syutliyka River; Bedechka River; Harmanliyska River; fishponds; Azmaka Dam near the village of Ivanovo, Harmanliysko	*Rhabdochona denudata* (Dujardin, 1845) Railliet, 1916
Kakacheva-Avramova (cited by Chunchukova & Kirin, 2020)	Tundzha River	*Caryophyllaeides fennica* (Schneider, 1902) Nybelin, 1922; *P. laevis*; *Rh. denudata*
Kirin, 2001	Kardzhali Reservoir	*Ichth. pileatus*, metacercariae; *Neogryporhynchus cheilancristrotus* (Wedl, 1855) Wedl, 1855 (syn. *Gryporhynchus cheilancristrotus* (Wedl, 1855)); *Rh. denudata*
Kirin et al., 2002	Arda River, Rabovo	*C. fennica*; *Schyzocotyle acheilognathi* (Yamaguti, 1934) Brabec, Waeschenbach, Scholz, Littlewood & Kuchta, 2015 (syn. *Bothriocephalus acheilognathi* Yamaguti, 1934); *Ligula intestinalis* (Linnaeus, 1758) Gmelin, 1790, plerocercoid; *Paradilepis scolecina* (Rudolphi, 1819), cysticerc; *Rh. denudata*
	Arda River, Madzharovo	*C. brachycollis*; *Sch. acheilognathi*; *Rh. denudata*
Kirin, 2003	Arda River	*C. brachycollis*; *C. fennica*; *Bathybothrium rectangulum* (Bloch, 1782) Lühe, 1902; *Ac. anguillae*; *Rh. denudata*
Kirin et al., 2013	Arda River	free of parasites
Chunchukova et al., 2019	Maritsa River (Plovdiv)	*Allocreadium isoporum* (Looss, 1984); *C. brachycollis*; *L. intestinalis*; *P. laevis*; *Rh. denudata*
Chunchukova & Kirin, 2020	Tundzha River	*Ac. anguillae*
Zaharieva et al., 2024	Luda Yana River (Svoboda)	*Raphidascaris acus* (Boch, 1779)
	Chepelarska River (Katunitsa)	free of parasites

Of the parasite species discovered in this study, the acanthocephalan *P. laevis* is pathogenic for fish and causes the disease pomphorhynchosis associated with intestinal damage in infected fish (Novakov et al., 2015). The metacercariae of the trematode *P. cuticola* cause the disease postodiplostomosis, which can lead to refusal to feed, rapid weight loss and death of fish (Rusev & Stratev, 2021).

CONCLUSIONS

The present study provides the first data on the component and infracommunity of the bleak from the Mechka River. Infection with six parasite species was detected - *P. cuticola*, Metacercariae; *Ichth. pileatus*, Metacercariae; *Ac. anguillae*; *P. laevis*; *Ps. tomentosa*; *Sch. petruschewskii*. The Parvomay (Debar) biotope is a new habitat for the six identified species of bleak parasites. *P. cuticola*, *Ps. tomentosa* and *Sch. petruschewskii* are reported for the first time from *Alb. alburnus* from the East Aegean region in Bulgaria. No parasite species pathogenic to humans were detected. Two of the identified parasite species - *P. laevis* and *P. cuticola*, cause diseases in fish that can affect fish populations. *P. laevis* has a high prevalence (P % = 46.30) in bleak from the Parvomay biotope (Debar). To protect fish resources, it is recommended that parasitological research in the freshwater ecosystem of the Mechka River be continued.

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RESEARCH ON THE MINERAL AND VITAMIN CONTENT OF BROWN TROUT MEAT DIFFERENTIATED FEED

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Abstract

Trout meat is rich in essential minerals and vitamins that contribute to human health. These nutrients are influenced by various factors, including breed, breeding system, season, age, and diet. Taking these factors into account, the aim of this study is to highlight how the quantity and quality of the feed that is supplied have an effect on the mineral and vitamin content of the meat of brown trout. The biological material was represented by 30 specimens of brown trout. At the end of the research, among the analysed minerals, the highest values were found in the case of phosphorus (P), which was in the range of 1775.83-2228.39 g.kg^{-1}, and copper (Cu) was in last place, with average values that varied in the range of 0.65-0.91 g.kg^{-1}. In terms of fat-soluble vitamins present in the meat, the highest concentration was of vitamin E, whereas vitamin D3 exhibited the lowest levels across all batches.

Key words*: brown trout, feed, mineral, vitamin content.*

INTRODUCTION

Brown trout *Salmo trutta* (Linnaeus 1758), also referred to as native or mountain trout, belong to the family of fish known as Salmonidae, being the most widespread species from the salmon family in Romania. It is widespread in the upper courses of mountain rivers and in mountain lakes; sometimes it is also present in watercourses in hilly areas and is highly appreciated by fishermen (Kaya & Erdem, 2009). Being a fish with highly appreciated meat (Kayim et al., 2011), is extensively cultivated in specialised trout farms, despite the preference for the more rapidly growing rainbow trout.

Fish meat has always been a staple product in human nutrition throughout the evolution of humanity, contributing significantly to the provision of necessary protein sources. Globally, 15% of eaten animal protein derives from fish and fish products being recommended for daily consumption (Pariser & Wallerstein, 1980; Novikov et al., 1997; Tocher et al., 2003; Osibona et al., 2009; Sabetian et al., 2012; Bissih, 2021; Mendivil, 2021; Chen et al., 2022), due to the fact that protein requirements of individuals can be met with only 400 grams of fish (Vranici et al., 2011).

The variations in the chemical composition of trout meat are influenced by a number of factors such as breed, genetic factors, sex, age, fishing season, environment, water salinity, temperature and its pH, feeding intensity, fodder type and quality, amount of natural food, breeding type (Shirai et al., 2002; Yeannesa & Almandos, 2003; Solvik and Rustad, 2005; Toppe et al., 2007; Hadzhinikolova & Atanasova, 2007; Erdem et al., 2009; Sabetian et al., 2012; Tkaczewska & Migdał; 2012; Teodorowicz 2013; Kaya et al., 2014; Sirakov, 2015; Siemianowska et al., 2016; Bayir et al., 2018; Rasul et al., 2021; Imtiaz et al., 2022; Sandu et al., 2023; Cocan et al., 2024).

Fish meat includes elevated levels of minerals, including phosphorus, calcium, magnesium, potassium, and substantial quantities of vitamins (Celik et al., 2008; Stoyanova, 2016; Mendivil, 2021) and their presence has biological significance, as numerous minerals participate in metabolic processes in all life forms (Tilami & Sampels, 2017), and their insufficiency can induce severe disorders in human body.

Minerals accumulate in trout meat not only from the administered feed but also from the natural environment through the gills and skin (Lall, 2002); however, their final content is influenced by environmental conditions and season (Bayir et al., 2018) and can even serve as an indicator of water contamination with heavy metals

(Alibabic et al., 2007; Alberto et al., 2021; Milošković et al., 2023).

MATERIALS AND METHODS

The aim of this paper was to assess the chemical composition in general of brown trout meat and, in particular, to highlight the mineral and fat-soluble vitamin content of it. There is available information regarding the meat quality of brown trout, but concerning the mineral and vitamin content, data from the specialised literature are limited.

Research took place in three trout farms from Suceava County in which different breeding and feeding strategies are applied.

In the first trout farm (F1), an extensive breeding system is applied, fish being raised in earth ponds, their feeding being based on the natural productivity of the ponds and an additional morning ratio of a conventional feed.

In the second farm (F2), a semi-intensive breeding system is used, trout's being bread similarly in earth ponds, being fed twice a day with a conventional feed.

At the third farm (F3), an intensive breeding system is practiced in concrete ponds, trout's being fed twice a day with a similar conventional feed as in the case of the second farm.

The chemical composition of the feed used in the feeding of trout from the three farms is presented in Table 1.

Table 1. Chemical composition of the used feed

Specification	Farm 1	Farm 2	Farm 3
Proteins (%)	42.00	40.00	42.00
Lipids (%)	12.00	22.00	22.00
Ash (%)	7.00	5.00	4.00
Cellulose (%)	2.7	3.0	2.00
Phosphorus (%)	0.8	1	-
Calcium (%)	0.7	0.7	-
Sodium (%)	0.2	0.2	-
Iron (mg/ kg)	40.0	40.0	-
Zinc (mg/ kg)	90.0	90.0	-
Copper (mg/kg)	5.0	6.0	-
Vitamin A (IE)	2500	10000	20000
Vitamin D3 (IE)	500	1500	2000
Vitamin E (mg)	100	200	200

As can be seen, there are differences between the main chemical constituents, especially with regard to lipid and vitamin content. In the case of the third feed, the mineral content was not stated on the label.

The biological material was of 30 brown trout, 10 fishes of both sexes were randomly picked from each farm during August-September of 2024 with a mass ranging from 215 to 381 grams. To determine the physical and chemical composition, samples were gathered from the fish muscles. Analyses were performed in duplicate.

Determination of the main chemical compounds was carried out as described by Nistor et al., 2014; 2019. Water and dry matter content was conducted using the drying method in an oven at +105°C until a constant weight is achieved. The determination of protein involves decomposing the analysed sample by heating it with sulphuric acid in the presence of catalysts, which reduces organic nitrogen to ammonium ions that can be quantified by distillation/titration. The equipment employed is the Kjeltec Auto 2300-Tecator, Sweden, which is a semi-automatic version of the crude protein Kjeldahl determination. The determination of lipid content was carried out using the Soxhlet method, which entails the extraction of fat from the analysed sample using petroleum ether with the Velp Scientifica SER 148 device.

Mineral determination was performed using a Shimadzu AA 6300 spectrophotometer in accordance with SR EN 14082/2003. 2 grams of samples were burnt at 550°C, dissolved, precipitated, filtrated and diluted to a desired volume, followed by aspiration into the air-acetylene flame of the atomic absorption spectrometer as described by Pagu et al. (2014), at different wavelength.

For phosphorus determination of, 1 g of sample was calcined in an oven at 550°C, hydrochloric acid solution was added for dissolution, filtered and made up to the mark with double-distilled water, introduced into the test tube together with the vanadium-molybdenum reagent. The absorbance measurement was made on a Shimadzu UVmini-1240 spectrophotometer at a wavelength of 430 nm by comparison with the reference solution in accordance with ISO 13730:1996 and SR ISO 2294:2009.

Determination of fat-soluble vitamins was carried out using a Shimadzu HPLC LC-20A equipped with analytical column. All samples underwent a 1:10 methanol dilution prior to filtration using a 0.2 µm filter. Analytical parameters for High-Performance Liquid

Chromatography (HPLC) were C18 5 µm x 4.6 mm ID x 250 mm, column temperature 40°C, mobile phase was acetonitrile: methanol 60:40 (v/v), flow rate 1 ml/min., injection volume 10 µl. Detection was at 265 nm, 280 nm and 325 nm.

The statistical analysis software utilized was SPSS. Mean, standard deviation, and statistical significance of the differences between batches were calculated.

RESULTS AND DISCUSSIONS

The quality of brown trout meat is determined not only by its sensory characteristics but also by its content of nutrients and micronutrients.

The chemical composition of brown trout depending on the farm system and feeding levels is presented in Table 2.

Table 2. Chemical composition (%) of brown trout meat depending on system and feeding ratio

Specification	Farm 1	Farm 2	Farm 3
Moisture	78.23±1.12^{c}	77.34±0.68^{b}	76.77±0.52^{a}
Dry matter	21.97±1.83^{c}	22.66±1.21^{b}	23.23±0.34^{a}
Proteins	18.3±0.76^{b,c}	17.77±0.32^{a}	17.65±0.21^{a}
Lipids	2.31±0.84^{a}	3.60±0.68^{b}	4.26±0.34^{c}
Ash	1.16±0.07^{a}	1.29±0.06^{a}	1.32±0.03^{a}

*Means in the same row with the same letter do not significantly differ at the level $p<0.05$.

It is observed that there are significant differences between all the major chemical constituents, except ash content, in the three systems of breeding and feeding applied on farms.

The dry matter content differed among the three breeding systems, with values spanning from 21.97% in the first farm to 23.23% in the third farm, suggesting that trout receiving two daily rations exhibited a higher dry matter content than those receiving a single ration. This result indicates that feeding frequency may influence the dry matter content.

Comparing the results to other research regarding brown trout (wild and in different breeding systems) it can be observed that in the dry matter case, are similar to those reported by Kayim et al. (2011), Nistor et al. (2014; 2019), Kaya et al. (2014), Sirakov (2015), Akhan et al., (2016), Barylo & Loboiko, (2018), Antão-Geraldes et al. (2018). However, they are lower than those documented by Koskela et al. (1997), Yeşilayer & Genç (2013), Bosco et al. (2013), Tilami et al. (2018), Erdem et al. (2020), and Alberto et al. (2021).

In the present study, the protein content ranged from 17.65% to 18.3%. Similarly, the protein content across the three farms was influenced by diet. However, in the case of Farm 2, despite the protein level of the feed being lower (40%) than that of Farm 3 (42%), the protein content of the trout meat was higher. Possible explanations for this discrepancy include differences in the type of breeding ponds (earth versus concrete) and/or the natural productivity of the basin. Despite receiving a single feed ration, the trout in the first farm displayed the highest protein content in their meat. This phenomenon may be attributed to the natural productivity of the pond and the occurrence of cannibalism among the trout.

The obtained results are higher than those reported by Koskela et al. (1997), and Kaya et al. (2014), similar to findings from several other studies, including those by Koskela et al. (1997), Kayim et al. (2011), Yeşilayer & Genç (2013), Nistor et al. (2014; 2019); Kaya et al. (2014), Sirakov (2015), Akhan et al. (2016), Barylo & Loboiko (2018), Antão-Geraldes et al. (2018), Tilami et al. (2018), Erdem et al. (2020), and Alberto et al. (2021). However, they are lower than those found by Bosco et al. (2013), and Akhan et al. (2016).

Lipids exhibited values ranging from 2.31% for samples from Farm 1 to 4.26% for samples from Farm 3, indicating that both the number of rations and the lipid content of the feed influence the final lipid content of the meat.

The results obtained are higher than those reported by Sirakov (2015), Akhan et al. (2016) and Antão-Geraldes et al. (2018) and, while they are similar to those of Kaya & Erdem (2009), Kayim et al. (2011), Yeşilayer & Genç (2013), Bosco et al. (2013), Nistor et al. (2014; 2019), Kaya et al. (2014), Akhan et al. (2016), Barylo & Loboiko (2018), Tilami et al. (2018), and Alberto et al. (2021). However, they are lower than those reported by Koskela et al. (1997), and Erdem et al. (2020).

Ash content was the only chemical parameter that did not differ significantly in all the experimental farms. The obtained results (1.16-1.32%) were similar to those researches mentioned above (1.09-1.6%), except the studies of Koskela et al. (1997), and Alberto et al. (2021).

Minerals are important for the proper functioning of the human body. They play key roles in various metabolic processes (Mishra, 2020). The mineral content of brown trout samples is presented in Table 3.

Table 3. Mineral content (mg.kg^{-1}) of brown trout meat depending on system and feeding ratio

Minerals	Farm 1	Farm 2	Farm 3
Calcium	220.73±0.45^{a}	247.88±0.35^{ab}	222.81±0.26^{bc}
Magnesium	134.69±1.55^{a}	231.62±1.63^{b}	168.12±1.72^{ac}
Sodium	156.53±1.47^{ab}	113.68±0.92^{ab}	96.35±0.79^{a}
Potassium	2623.1±1.33^{a}	2788.6±1.23^{bc}	2438.1±1.66^{c}
Zinc	9.7±0.92^{a}	7.1±0.83^{ab}	6.3±0.41^{bc}
Iron	16.68±1.15^{a}	5.28±0.23^{ab}	2.84±0.41^{abc}
Copper	0.71±1.51^{a}	0.91±1.23^{bc}	0.65±1.33^{b}
Phosphorus	1775.83±1.08^{a}	2228.39±1.66^{ab}	1994.1±1.15^{abc}

*Means in the row with different letters are significantly different at the level $p<0.05$.

As it can be observed, there are significant differences between elements depending of farming systems and feeding ratio, which is in correlation with the observation of Siemianowska et al., 2016.

Calcium is an essential mineral for the development, growth, and maintenance of bones and teeth and carries out many important roles in blood clotting and circulation, regulating muscle contractions, heart rhythm, and normal functioning of many enzymes.

The calcium levels were between 220.73 mg.kg^{-1} at Farm 1 and 247.88 mg.kg^{-1} at Farm 2, which are higher than what Alibabic et al. (2007), Kayim et al. (2011), Sirakov (2015), and Bayir et al. (2018) reported. However, these results are similar to those presented by Tidball et al. (2017), for wild brook trout. For rainbow trout raised in various systems, calcium levels reported by other authors ranged from 2.66 mg.kg^{-1} (Baskaya et al., 2023) to 632 mg.kg^{-1} (Gokoglu et al., 2004).

Magnesium, similar to calcium, is an essential macronutrient, playing an important role in nerve transmission, neuromuscular conduction, blood pressure, muscular contraction, and is even a cofactor in proteins, DNA, and RNA synthesis.

The magnesium content of brown trout meat ranged from 134.69 mg.kg^{-1} in samples from Farm 1 to 231.62 mg.kg^{-1} in samples from Farm 2. These values are comparable to those reported by Kayim et al. (2011), and Sirakov (2015) but lower than the values obtained by Bayir et al. (2018). Similar variation in data can be observed for other trout species. Tidball et al. (2017), found that wild brook trout had a magnesium level of 280 mg.kg^{-1}, while at rainbow trout magnesium levels ranged between 59.5 mg.kg^{-1} (Brucka-Jastrzebska & Kawczuga, 2011) up to 409 mg.kg^{-1} (Gokoglu et al., 2004).

Sodium is one of the important electrolytes in the body and plays a variety of fundamental roles in the body, being essential for the body's acid-base and fluid balance, being an active transport mechanism, and, along with potassium, is responsible for balancing nerve stimulation and muscle contraction.

In this study, the values obtained varied from 96.35 mg.kg^{-1} in samples from Farm 3 to 156.63 mg.kg^{-1} in samples from Farm 1, which are lower than those reported in other studies for brown trout. For wild brook trout, Tidball et al. (2017), found a sodium content of 450 mg.kg^{-1}, whereas for rainbow trout, values ranged from 19.53 mg.kg^{-1} (Baskaya et al., 2023) to 718.2 mg.kg^{-1} (Siemianowska et al., 2016).

Potassium is an essential mineral, playing a fundamental role in various metabolic processes, including regulating fluid and electrolyte balance, nerve signalling, cell integrity and muscle contractions.

In terms of potassium content in brown trout meat, results ranged from 2438.1 mg.kg^{-1} in samples from Farm 3 to 2788.6 mg.kg^{-1} in those from Farm 2. Other researchers reported lower values, such as Bayir et al. (2018), or similar results, such as Kayim et al. (2011), and Sirakov (2015). However, these results are substantially lower than those found for other trout species.

Even though zinc plays an essential role in our biological system in skin and nail health, preventing retarded growth, loss of taste, together with vitamin A, forms melanin, immune function and other conditions (Sivaperumal et al., 2007), higher levels of zinc may lead to health problems (Kayim et al., 2011).

Zinc content in this study varied from 6.3 mg.kg^{-1} at Farm 3 to 9.7 mg.kg^{-1} at Farm 1. These results are higher than those reported by Vitek et al. (2007), and Alberto et al. (2021), and similar to findings by Alibabic et al. (2007), and Kayim et al. (2011). However, they are lower compared to the values observed by Sirakov (2015), Bayir et al. (2018), and Mona et al. (2011). Similar variations in data have been

noted for other trout breeds. Tidball et al. (2017), found that wild brook trout had a zinc content of 5.5 mg.kg^{-1}, while rainbow trout had zinc levels ranging from 2.96 mg.kg^{-1} (Karimian-Khosroshahi et al., 2016) to 19.1 mg.kg^{-1} (Apines-Amar et al., 2004).

Copper is a vital mineral with several important functions in the human body. It is involved in red blood cell production, nerve cell health, and immune system support and is a cofactor for several enzymes. Furthermore, copper plays a role in collagen formation, iron absorption, and energy production. It possesses anti-infectious, antiviral, and anti-inflammatory properties, aiding the body in combating microbial threats.

Copper levels in this study ranged from 0.65 mg.kg^{-1} at Farm 3 to 0.91 mg.kg^{-1} at Farm 2. These findings are higher than those reported by Linde et al. (2004), Vitek et al. (2007), Mona et al. (2011), and Can et al. (2012), and are similar to results from Kayim et al. (2011), and Alibabic et al. (2007). However, they are lower than those reported by Sirakov (2015), Bayir et al. (2018), and Alberto et al. (2021). A similar variability is evident for rainbow trout, with data ranging from 0.05 mg.kg^{-1} (Bajc et al., 2005) to 8.19 mg.kg^{-1} (Celik et al., 2008).

Iron is crucial for human health, being essential for producing haemoglobin, a protein in red blood cells that carries oxygen throughout the body. Iron also supports various vital functions, including energy production, concentration, and gastrointestinal processes.

The iron content of the analysed samples exhibited significant variation: at Farm 1, the average value was 16.68 mg.kg^{-1}; at Farm 2, was 5.28 mg.kg^{-1}; at Farm 3, was 2.84 mg.kg^{-1}. This variability is consistent with findings from other studies concerning brown trout. For brook trout, Tidball et al. (2017), reported a value of 3.8 mg.kg^{-1}, while Varol et al. (2017), noted a range of 2.53 mg.kg^{-1} to 13.29 mg.kg^{-1} for rainbow trout.

Phosphorus is an essential mineral vital for several bodily functions, being a key component of bones and teeth, supporting energy production and muscle function. It also contributes to DNA structure, ATP synthesis, and various cell signalling pathways.

The phosphorus content of the analysed samples ranged from 1775.83 mg.kg^{-1} on Farm 1 to 2228.39 mg.kg^{-1} at Farm 2 samples. These results are higher than those reported by Alibabic et al. (2007), and Kayim et al. (2011), and are similar to findings by Sirakov (2015), Bayir et al. (2018), and Tidball et al. (2017), who reported a value of 2460 mg.kg^{-1} for brook trout. In the case of rainbow trout phosphorus content, it ranged from 1104 mg.kg^{-1} (Pagu et al., 2014), to 4640 mg.kg^{-1} (Apines-Amar et al., 2004).

Fat-soluble vitamins (A, D3, E, and K2) are crucial for human health. They're absorbed alongside dietary fats and play key roles in vital processes. Vitamin A supports vision, vitamin D is essential for bone health, vitamin E acts as an antioxidant, and vitamin K2 is important for blood clotting. These vitamins contribute to immune regulation and mental health. Unlike water-soluble vitamins, which the body excretes, excessive intake of these vitamins, stored in the body's fat tissues, can lead to toxicity.

The values of A, D3, E, K2 vitamins are shown in Table 4.

Table 4. Mineral content (µg.100 g^{-1}) of brown trout meat depending on breeding system and feeding ratio

Vitamins	Vitamin A	Vitamin D3	Vitamin E	Vitamin K2
Farm 1	14.51±2.46	11.69±3.48	1551.03±3.64	509±2.17
Farm 2	15.66±1.48	12.77±3.88	2156.66±1.74	692.64±0.52
Farm 3	37.34±0.15	17.66±0.21	2235.45±0.65	878.33±0.98

All analysed samples are sources of fat-soluble vitamins. The values obtained vary according to the rearing system and daily feed ration, with the highest values recorded in samples from Farm 3 and the lowest from Farm 1.

No research specifically addressing the vitamin content of brown trout was found; thus, comparisons will be drawn with studies related to rainbow trout.

In terms of vitamin A, results ranged from 14.51 µg.100 g^{-1} to 37.34 µg.100 g^{-1}. Similar findings were reported by Stancheva et al. (2010; 2014), and Harlioğlu (2012), although these were higher than those noted by Dias et al. (2003). Nonetheless, the results obtained are lower than

those reported by Dobreva et al. (2017), and Ural et al. (2017).

The content of vitamin D3 in brown trout meat was found to range from 11.69 µg.100 g^{-1} to 17.66 µg.100 g^{-1}. These resuls are comparable to those reported by Dias et al. (2003), Stancheva et al. (2010; 2014), Harlioğlu (2012), and Dobreva et al. (2017); however, they are lower than the values reported by Ural et al. (2017).

The vitamin K content in brown trout meat ranged from 1151.03 µg.100 g^{-1} to 2235.45 µg.100 g^{-1}. These values are higher than the 392 µg.100 g^{-1} reported by Harlioğlu (2012), but lower than the findings of Ural et al. (2017), which ranged from 5220 µg.100 g^{-1} to 13760 µg.100 g^{-1}.

The vitamin E content was found to be the highest in brown trout meat with values ranging from 1551.03 µg.100 g^{-1} in case of Farm 1 samples to 2235.45 µg.100 g^{-1} at Farm 3 samples. This observation aligns with findings from other researchers regarding rainbow trout (Dias et al., 2003; Stancheva et al., 2010, 2014; Harlioğlu, 2012; Dobreva et al., 2017; Ural et al., 2017).

CONCLUSIONS

The investigated brown trout in this study were characterised by a good nutritional quality, a high amount of protein, mineral and vitamin content and a low level of lipid content.

The nutritional profile of fish varies depending of farming practices, and dietary differences.

Breeding type and feed influence the mineral content of brown trout meat. According of this study, fish rearing in semi-intensive breeding farm system contained higher mean concentrations of most of the mineral elements compared to trout from the other farms.

Brown trout meat is an important source of phosphorus and potassium in the human diet, providing a significant proportion of the daily recommended intake of these nutrients.

The addition of vitamins to fish feed increases the vitamin content found in the meat. The vitamin E content found in brown trout meat highlights its nutritional significance.

Future research concerning the mineral and vitamin content of trout meat are of great interest because there are insufficient data on this matter.

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CARABID FAUNA IN AGRICULTURALLY MODIFIED LANDSCAPES: A CASE STUDY OF THE ARGEȘ RIVER CATCHMENT, ROMANIA, FROM HEADWATERS TO FLOODPLAINS

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Abstract

Riparian zones represent valuable riverine habitats that contribute to regional biodiversity and promote valuable ecosystem services within their catchments. Land use changes historically impacted riparian areas within most agrarian catchments, leading only to small land gains at high cost of ecosystem service loss. Ground beetles represent validated bioindicators and an important group of predators acknowledged as valuable cross-ecosystem trophic links and pest control agents. We studied the community composition of ground beetles along a land use gradient within the riparian zones of a moderately impacted agricultural catchment. We emphasize the importance of riparian forested buffers for sustainable agriculture through the support of multiple pest predators and the promotion of higher biodiversity across agrarian landscapes.

Key words*: agricultural catchment, community composition, ground beetles, riparian buffers, sustainability.*

INTRODUCTION

Riparian zones are ecotones that connect lotic and adjacent terrestrial ecosystems across the different landscapes of large catchments (González et al., 2017; Naiman & Decamps, 1997). These riverine habitats are documented to possess distinct (Sabo et al., 2005) and often more diverse biotic communities compared to their surrounding areas (Naiman et al., 1993; Ramey & Richardson, 2017). In many instances, riparian zones have been shown to shelter rare and vulnerable species (Ramey & Richardson 2017; Tockner & Ward, 1999), further highlighting their importance for biodiversity conservation at local and regional levels (González et al., 2017; Zhang et al., 2023).

Historically, the increased demand for agricultural land led, in many cases, to the conversion of riparian areas, formerly perceived as unproductive habitats, into crops or pastures (Clerici et al., 2014; Fang et al., 2024). Land use changes have significantly affected riparian zones and their biodiversity, particularly in developed countries and continue to do so in developing and low-income regions (Hanna et al., 2020; Tockner & Stanford, 2002). More recent concepts such as green-blue infrastructure and sustainable agriculture led to increased awareness and conservation of wetlands such as riparian habitats (Burdon et al., 2020; Kohsaka & Uchiyama, 2021). Nowadays, an increasingly solid body of work advocates for the use of forested buffers as ecosystem features that facilitate sustainable practices and efficient ecosystem management across agricultural catchments (Burdon et al., 2020; Graziano et al., 2022).

Among riparian biota, ground beetles (family Carabidae) represent one of the most researched taxa (Ramey & Richardson, 2017), in part, due to their roles as cross-ecosystem trophic links (Murgu & Rîșnoveanu, 2023) and herbivory control agents (French et al., 2001, Murgu, 2023) and, in part due to their diversity and ubiquity in riverine habitats (Ramey &

Richardson, 2017). Conjointly, the strong habitat affinity and sensitivity to environmental conditions exhibited by carabids have made them commonly used biotic indicators (Januschke et al., 2011; Murgu & Rîșnoveanu, 2023). The pragmatic use and conservation of ground beetles for ecological assessment, pest control and for other ecosystem services they provide, as well as for scientific and moral purposes requires, first and foremost, an extensive knowledge of the taxa in the areas where sustainable ecosystem management is desired.

The Argeș River is one of the main tributaries of the Danube River in the lower part of its course, its catchment being one of the largest in the south of the Carpathians. More than half of its total area of 125 km^2 has been converted to agricultural lanscapes for crops, pastures, and orchards, with activity levels of varying intensites (Popescu et al., 2021). The carabid biodiversity of the Argeș cathcment has been addressed before throughout various research articles and monographs to different extents and degrees of complexity (Barloy & Prunar, 2010; Lotrean, 2010; Lotrean, 2012; Lotrean & Manu, 2017; Popescu, 2021; Teodorescu, 2010; Vizitiu et al., 2022).

However, to the best of our knowledge, the ground beetle communities of the Argeș river catchment have not been investigated, at least not for a long time, at the same spatial extent that we did, from headwater streams to the lowlands, across a diverse landscape and during a continuous multiseasonal period. In this research, we investigate the carabid fauna of the middle section of the Argeș catchment and aim to contribute to its comprehensive knowledge and to further emphasize the importance of riparian buffers in agricultural landscapes as shelters for rare and useful species which increase regional biodiversity and promote sustainable agricultural practices.

MATERIALS AND METHODS

Sampling of invertebrates took place monthly, from May to November of 2018 within the middle catchment of the Argeș River, in the territory of the Argeș and Dâmbovița counties in Romania (Figure 1).

The selected catchment serves as a good representation of both the sylvo-steppe landscapes in southeastern Europe and moderately modified agricultural catchments, as approximately 56% of its total area was converted for this purpose (Popescu et al., 2021).

Ground beetles were sampled following an altitudinal and anthropic impact gradient, from four types of riparian habitats (henceforth called site types): forest (F), unbuffered (I), buffered (R) and matrix (IC) and 17 sites (Figure 1, Table 1) located mostly along low order streams, between the 1st and 3rd order according to the Strahler classification (Strahler, 1957). All sampling sites were identified and characterized in the BioDivERsA Crosslink project (for details see Burdon et al., 2020 and Popescu et al., 2021).

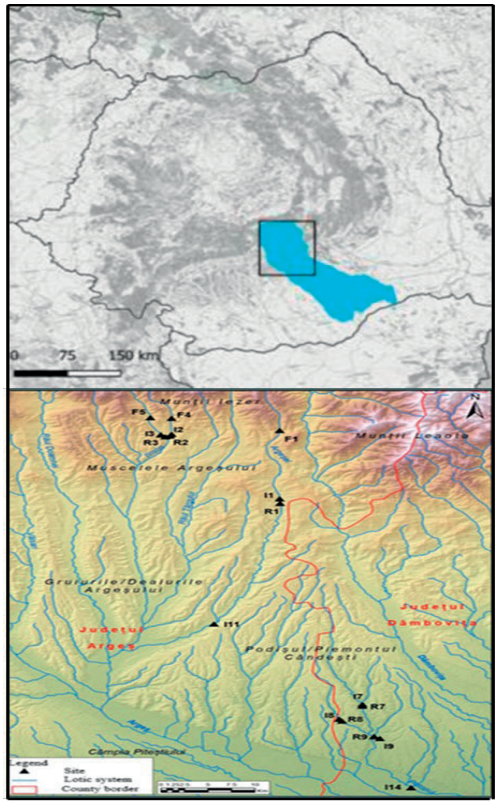

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Table 1. Location of sites within the Argeș Catchment: F = forest; I = unbuffered, R = buffered, IC = matrix

Site	Lotic system	Strahler order	Altitude (m.a.s.l.)	Region
F1	Argeșel	1	795	Getic Subcarpathians
F4	Brătioara	1	720	Iezer-Păpușa Mountains
F5	Bratia	1	730	Iezer-Păpușa Mountains
I1	Argeșel	1	570	Getic Subcarpathians
R1	Argeșel	1	560	Getic Subcarpathians
I2	Brătioara	1	655	Getic Subcarpathians
R2	Brătioara	1	645	Getic Subcarpathians
I3	Bratia	1	649	Getic Subcarpathians
R3	Bratia	1	638	Getic Subcarpathians
I7	Potop	2	262	Getic Plateau
R7	Potop	2	258	Getic Plateau
I8	Strâmbul	2	265	Getic Plateau
R8	Strâmbul	2	260	Getic Plateau
I9	Potop (Aval)	2	230	Getic Plateau
R9	Potop (Aval)	2	229	Getic Plateau
I11	Argeșel	2	364	Getic Plateau
I14	Potop	3	192	Getic Plateau

Forest sites were placed at the highest upstream locations in undisturbed deciduous forest landscapes and were characterized by extensive lengths and widths of native arboreal vegetation as well as rocky substrates and thin layers of soil. Paired unbuffered and buffered sites were located downstream along the river continuum, usually in slightly hilly or flat terrain within landscapes modified by agriculture. Unbuffered sites, located upstream of their counterparts, were characterized as open habitats that lacked arboreal vegetation, dominated by grasses, herbs and occasional shrubs. Buffered sites were characterized by woody riparian vegetation with a minimal length of 50 m and widths at least 2 or 3 times wider than the wetted stream channel. The proximity between the paired sites was intentional, to allow for a viable comparison, based on the level of land use impact. The matrix sites were the furthest downstream, affected by cumulative impact from upstream and located in intensely agriculturally converted areas from the floodplains. They were selected to represent the highest level of impact along the gradient. These sites were dominated solely by grasses that bordered crops or pastures with intensive land use regimes. Three sampling plots were established on each bank, for a total of six plots per site, to cover existing heterogeneity as well as to provide replication in case of inherent sample loss or deterioration. Ground beetles were collected with pitfall traps, half-filled with ethylene glycol and usually emptied fortnightly, depending on the weather. Samples were collected at three distances from the stream within each site (1, 5 and 15 m), with a total of 18 traps per site, to accurately assess the diversity within the studied communities as some species exhibit stronger aggregative responses towards water than others. Upon collection, arthropods were transferred to plastic containers filled with alcohol and transported to the laboratory where they were stored in a cold room and identified as soon as possible based on the available taxonomic keys and other related resources (Ciubuc, 2021; Gîdei & Popescu, 2012; Klimaszewski & Watt, 1997; Luff, 2007; Makarov, 1994). Functional traits of each species were also gathered from the available literature (Ciubuc, 2021; Gerisch, 2014; Gîdei & Popescu, 2012; Klimaszewski and Watt, 1997; Luff, 2007; Makarov, 1994) to allow for a better assessment of how carabid communities change in relation to impact and for more detailed discussions.

To account for variation in exposure days and loss of traps within each site we divided the sum of daily catch rates (individuals/day) type by the total corresponding number of recovered traps ($\Sigma_{\text{individuals/day}}/\#_{\text{recovered traps}}$). We calculated the relative frequency and the relative abundance of each carabid species sampled within each of the sampled site types. The relative abundance (%) of a species within a site type was calculated as: $\Sigma_{\text{daily catch rates}}$ of a certain species/$\Sigma_{\text{daily catch rates}}$ of all sampled carabids within the assessed site type * 100. The relative frequency (%) of a species within a site type was calculated as: # of samples with confirmed presence of a certain species/# of samples collected from the assessed site type. We presented these values in increased order in the form of Whittaker plots, ranking species from the least abundant and rare to the most dominant and frequent. We opted for this type of approach since we consider it effective and clear to highlight the particularities of the carabid communities between the assessed site types.

RESULTS AND DISCUSSIONS

In total, at catchment level, we sampled carabids belonging to 14 subfamilies and 36 genera. We were able to identify 60 species (89.8% of the total carabids sampled), while some individuals were identified only to genus level, mostly due to their deterioration during sampling or difficulties in their group taxonomy, such as the case of *Bembidion* or *Ocys* (Table 2).

Table 2. List of carabid taxa identified within the studied sites: Habitat specialization: E = eurytopic, S = stenotopic; Pest predator: Ap = aphids, Co = coleopterans, Di = dipterans, Le = lepidopterans, Ss = slugs and snails, W = weeds; Status: E = endemic, P = protected, R = rare (For site code explanation see Table 1)

Subfamily	Species	F1	F4	F5	I1	I2	I3	I7	I8	I9	R1	R2	R3	R7	R8	R9	I11	I14	Habitat specialization	Pest predator	Status
Brachininae	*Brachinus crepitans* (Linnaeus, 1758)														X			X	E		
Broscinae	*Broscus cephalotes* (Linnaeus, 1758)		X	X			X			X									S	Co	
Carabinae	*Calosoma sycophanta* (Linnaeus, 1758)													X					S	Le	R
	Carabus coriaceus (Linnaeus, 1758)		X		X			X	X	X	X		X	X		X			E	Co	
	Carabus cancellatus (Illiger, 1798)		X	X	X						X		X	X					E	Co, Le	
	Carabus clatratus (Linnaeus, 1761)					X	X												S		R
	Carabus convexus (Fabricius, 1775)		X		X	X		X			X	X	X	X					S		
	Carabus glabratus (Paykull, 1790)										X								S		
	Carabus granulatus (Linnaeus, 1758)		X	X	X						X		X	X		X	X		E	Co, Ss	
	Carabus intricatus (Linnaeus, 1761)										X								S		R
	Carabus ullrichi (Germar, 1824)		X													X			E		
	Carabus variolosus (Fabricius, 1787)	X	X	X			X					X							S		P
	Carabus violaceus (Linnaeus, 1758)	X	X	X	X	X		X	X	X	X		X	X			X		E	Co, Ss	
	Cychrus caraboides (Linnaeus, 1758)	X	X										X	X	X				E		
Chlaeniinae	*Callistus lunatus* (Fabricius, 1775)													X					S		R
	Chlaenius nigricornis (Fabricius, 1787)			X			X						X				X	X	S		
	Chlaenius nitidulus (Schrank, 1781)					X	X	X	X	X		X		X		X		X	S		
	Chlaenius spoliatus (P.Rossi, 1792)					X	X												E		
	Chlaenius vestitus (Paykull, 1790)		X	X		X	X							X					E		
Cicindelinae	*Cicindela germanica* (Linnaeus, 1758)				X														S		
Harpalinae	*Diachromus germanus* (Linnaeus, 1758)																	X	E		R
	Harpalus griseus (Panzer 1796)					X		X	X		X	X		X				X	S	Ap, Di, Ss, W	
	Harpalus rufipes (De Geer, 1774)			X		X	X	X					X	X		X		X	E	Ap, Co, Di, Ss, W	
	Harpalus affinis (Schrank, 1781)							X	X				X						S	Ap, Di, W	
	Harpalus sp.		X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	E	Ap, W	
	Stenolophus teutonus (Schrank, 1781)													X					E	W	
Lebiinae	***Syntomus sp.***		X	X		X						X				X			E		
Liciniinae	*Badister bipustulatus (Fabricius, 1792)*				X														E		
	Licinus depressus (Paykull, 1790)				X							X	X				X		S	Ss	
Nebriinae	*Leistus piceus* (Frölich, 1799)													X					S		R
	Nebria brevicollis (Fabricius, 1792)	X	X			X				X	X		X	X				X	E	Ap	
	Notiophilus palustris (Duftschmid, 1812)			X									X						E		
Panagaeinae	*Panagaeus cruxmajor* (Linnaeus, 1758)																X	X	S		R
Platyninae	*Agonum piceum* (Linnaeus, 1758)	X																	E	W	
	Agonum sexpunctatum (Linnaeus, 1758)	X		X			X				X						X	X	E		
	Agonum viridicupreum (Goeze, 1777)	X					X	X	X										S		
	Anchomenus dorsalis (Pontoppidan, 1763)							X						X				X	E	Ap	
	Oxypselaphus obscurus (Herbst, 1784)	X										X							E		
	Platynus assimilis (Paykull, 1790)	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	E		
	Platynus sp.		X	X									X				X		E		
Pterostichinae	*Abax parallelepipedus* (Piller & Mitterpacher, 1783)	X	X								X					X			E	Di, Ss	
	Abax sp.		X		X						X					X			E		
	Amara aenea (De Geer, 1774)	X	X		X		X		X			X	X	X		X	X	X	E	Co, W	
	Amara ingenua (Duftschmid, 1812)	X											X					X	E	Co, W	
	Amara nitida (Sturm, 1825)													X		X		X	E	Co, W	
	Amara sp.	X	X		X				X	X						X	X		E	Co, W	
	Calathus fuscipes (Goeze, 1777)						X							X		X		X	E	Ap, W	
	Calathus melanocephalus (Linnaeus, 1758)	X	X	X	X	X	X	X	X	X	X			X		X		X	E		
	Calathus piceus (Marsham, 1802)						X						X						S		
	Molops piceus (Panzer, 1793)		X		X														S		
	Poecilus cupreus (Linnaeus, 1758)	X	X	X	X	X	X	X		X	X	X	X		X	X	X	X	E		
	Poecilus sericeus (Fischer von Waldheim, 1824)								X										E		
	Poecilus versicolor (Sturm, 1824)	X				X	X						X						E		
	Pterostichus macer (Marsham, 1802)	X			X				X		X	X	X			X			E	Ap, Co	
	Pterostichus madidus (Fabricius, 1775)							X					X				X		E	Ap, Co, Ss	
	Pterostichus melanarius (Illiger, 1798)	X			X	X	X					X	X					X	E	Ap, Co, Di, Le	
	Pterostichus niger (Schaller, 1783)	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	E	Co	
	Pterostichus nigrita (Paykull, 1790)		X		X			X	X		X	X	X	X			X	X	E	Co	
	Pterostichus sp.				X		X						X	X	X		X	X	E	Co	
	Pterostichus vernalis (Panzer, 1795)							X								X		X	E	Co	
	Stomis pumicatus (Panzer, 1796)												X						E		
Scaritinae	*Clivina fossor* (Linnaeus, 1758)		X							X						X		X	E	Co	
Trechinae	*Asaphidion caraboides* (Schrank, 1781)							X				X	X			X	X	X	E	Co	
	Bembidion lampros (Herbst, 1784)	X	X	X		X	X					X	X			X		X	E	Ap, Co, Di, Le	
	Bembidion sp.	X				X	X	X	X			X		X		X			E	Co	
	Ocys sp.							X				X							E		
	Tachys sp.			X						X		X						X	E		
	Trechus carpathicus (Rybinsky, 1902)	X																	S		E
	Trechus pilisensis (Csiki, 1918)	X														X			E	W	R

From all sampled taxa, carabids accounted for 11% of the total individuals sampled per day (ind/day) from buffered (0.18 ± 0.37 carabids/day of 1.62 ± 2.47 epigean invertebrates/day) and matrix sites (0.20 ± 0.36 carabids/day of 1.70 ± 2.13 invertebrates/day) and 8% of unbuffered (0.12 ± 0.23 carabids/day of 1.36 ± 2.24 invertebrates/day), respectively 6% of forest sites (0.11 ± 0.21 carabids/day of 1.62 ± 2.02invertebrates/day) (Figure 2).

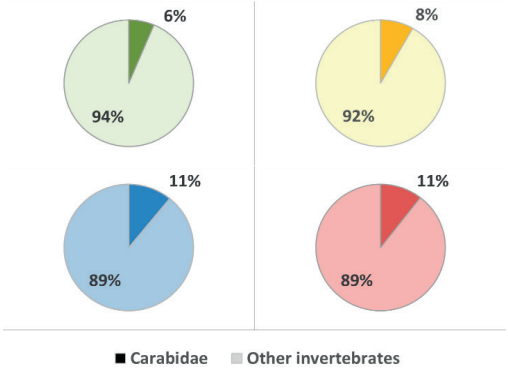


Figure 2. Proportion of carabids among total epigean invertebrates sampled at each site type: green = forest; orange = unbuffered, blue = buffered, burgundy = matrix

Buffered sites supported the highest number of species (48), followed by unbuffered (40), forest (38) and lastly matrix sites (28). Of all the ground beetle species sampled, 18 were found in all site types, 24 in three site types, 14 in two site types and 16 in just one site type, mostly in buffered (Table 2). None of the species were present in all sites, though 3 of them were highly widespread within the catchment, namely *Pterostichus niger* and *Platynus assimilis* in 16 sites and *Poecilus cupreus* in 15 sites (Table 2). Conversely, 13 species had their presence confirmed solely in one site (Table 2). As such, 7 species were found in just a single buffered site, 3 in a single unbuffered site, 2 in just a forest site and 1 just in one matrix site (Table 2). The Pterostichinae subfamily was by far the most diverse, with 18 identified species from 7 genera (Table 2). It is likely that more species for this subfamily can be found in our study area since a few individuals remained identified only to genus level. Carabids of this subfamily were the most widespread and frequently occurring throughout the different site types and site assessed, while also the most abundant in three of the four site types assessed. Species *Pterostichus niger* and *Poecilus cupreus* were some of the most frequently sampled species (Figure 3), with presence repeatedly confirmed in most sites. Both species were also among the most abundant regardless of site type (Figure 4). *P. niger* ranked second in buffered and third in matrix sites and had the third highest abundance at catchment level (Figure 4).

P. cupreus was, overall, the most abundant species, dominant in all site types except buffered, with relative abundances more than two-fold higher compared to the second most dominant taxa in all the cases (Figure 4). Oppositely, the rarest species of the subfamily were *Poecilus sericeus* and *Stomis pumicatus.* Each of the two species was present in just one site (Table 2) and both with very reduced relative abundances (Figure 4). Generally, Pterostichinae species were present at low abundances throughout the entire catchment, with only one species found to be subdominant, namely *Pterostichus nigrita,* in buffered and matrix sites (Figure 4). However, no rare, endangered or protected species belong to this subfamily were present within our study catchment.

Subfamily Carabinae was the second most diverse, represented by 12 species from 3 genera, with all but two belonging to the genus *Carabus* (Table 2). Of the genus, 4 species were recorded in just one site type, all but one in buffered sites, and each found in just one site: *Calosoma sycophanta* in R7 and *Carabus glabratus* along with *Carabus intricatus* in R1. By the contrary, *Carabus clatratus* was found only in unbuffered sites: I2 and I3. Rarities aside, most species were found in all or multiple site types and sites, the majority of them with reduced relative abundance, except for: *Carabus violaceus*, *Carabus granulatus*, *Carabus coriaceus* and *Cyhrus caraboides* (Figure 4). Notably, individuals of the vulnerable and protected species *Carabus variolosus* (Nitzu et al., 2021) were also observed throughout different site types and locations. However, they were not sampled in large numbers, with the highest relative abundance and frequency in forested sites (Figures 3 and 4). In addition, *C. sycophanta* is another species of the subfamily that is listed as vulnerable by the Romanian Red

Book of Invertebrates and a rarity in our samples, yet, at present, no enforced protection measures exist for it (Nitzu et al., 2021).

Six species from four genera were found to belong to the Platyninae subfamily (Table 2), thus making it the third most diverse within our area of study. *Platynus assimilis* was almost ubiquitous, missing from just one site. The species was dominant and most frequent in buffered sites, with relative abundances twice as high as *P. niger*, the second most dominant species (Figures 3 and 4).

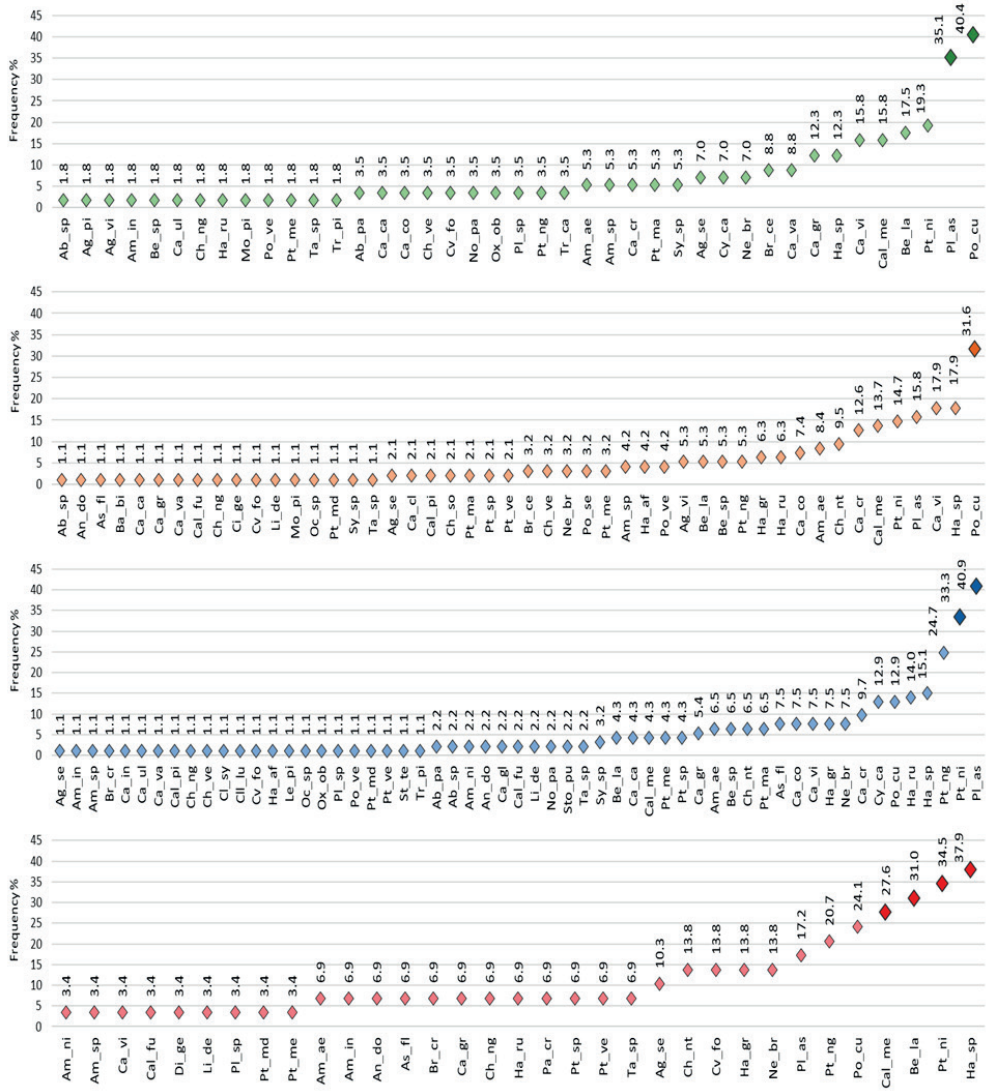


Figure 3. Relative frequency of species at site types: green = forest; orange = unbuffered, blue = buffered, burgundy = matrix

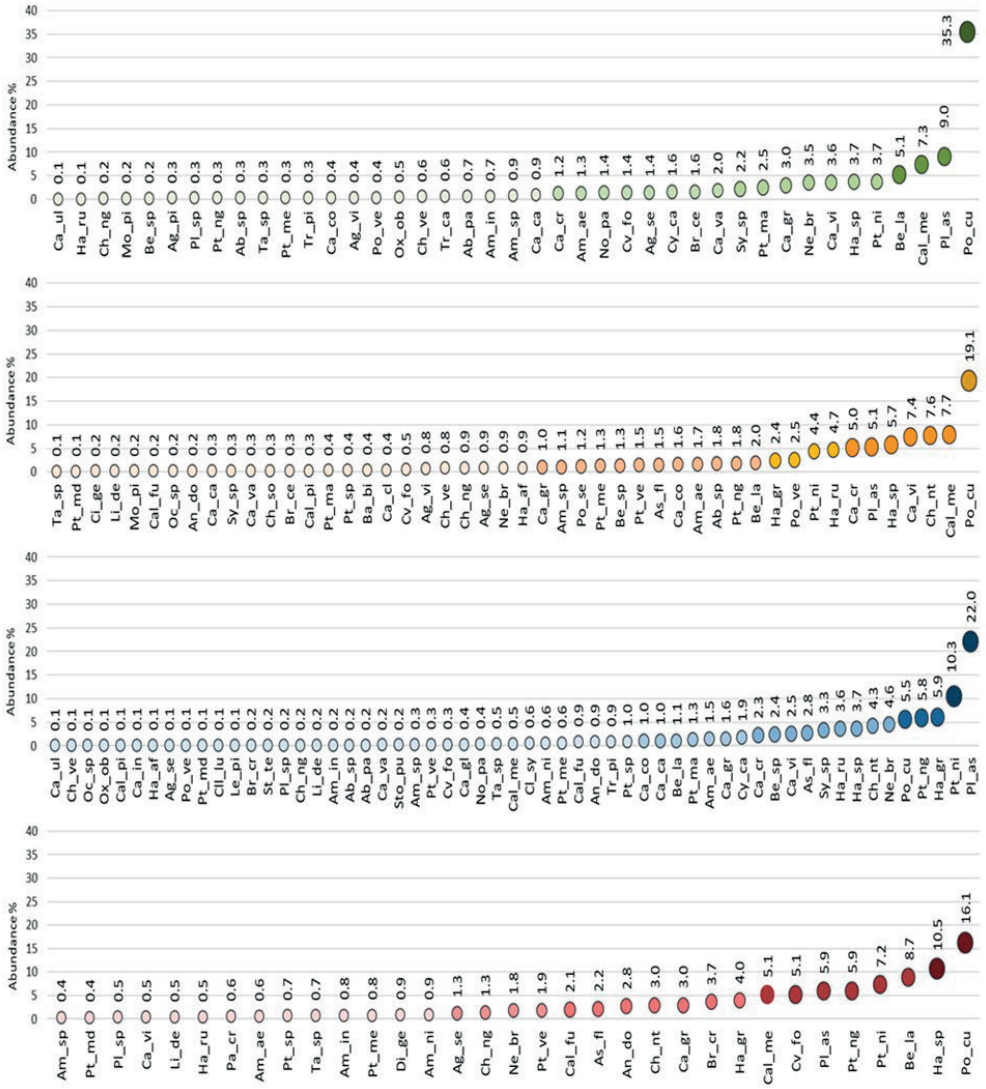


Figure 4. Percentage of species abundance at site types:
green = forest; orange = unbuffered, blue = buffered, burgundy = matrix

It showed high relative abundances in the other site types and occupied the second position in forest, fifth in matrix and sixth in unbuffered sites. Conversely, species like *Agonum piceum* and *Oxypselaphus obscurus* had reduced frequencies and abundances in communities, with the first present in just one forest site and the second in one forest and one buffered site (Figures 3 and 4). With the exception of *P. assimilis*, all species showed reduced relative abundance, with the notable exception of *Anchomenus dorsalis* in matrix sites where it could be classified as subdominant (Figure 4).

Subfamily Trechinae was represented by five genera from which we could identify four species, while most individuals from genus *Bembidion*, *Ocys* and *Tachys* remained unidentified (Table 2). The most widespread, frequent and abundant species was *Bembidion lampros*, with *Asaphidion caraboides* ranking second within the group (Figures 3 and 4).

Species *Trechus carpathicus* and *Trechus pillisensis* had very sporadic and spatially restricted occurrences, as well as very reduced relative abundances (Table 2, Figures 3 and 4). *T. carpathicus* is a rare, endemic and stenotopic forest species from the Romanian and Ukrainian Carpathian range (Ciubuc, 2021), found during our survey in only one forest site (Table 2). Its endemic congeneric species, *T. pillisensis* was found in one forest and one buffered site, however in both locations with reduced numbers.

Also represented by five species was the Harpalinae subfamily. Three species belonged to genus *Harpalus*, one to *Diachromus* and one to *Stenolophus*, while some individuals remained identified just to the eponymous genus (Table 2). The majority of species were present in multiple site types and sites, with the exception of *Biachromus gemanus* and *Stenolophus teutonus*, with the first reported only in site I14 (matrix) and the latter only in site R7 (buffered) (Table 2), both with very reduced relative abundance and frequency (Figures 3 and 4). *Harpalus griseus* and *Harpalus rufipes* had the highest recorded relative abundance and frequency of occurrence among the species in this subfamily. Both species were subdominant, in most buffered sites, but also in matrix and unbuffered ones, in the case of the former, respectively of the latter.

The Chlaeniinae subfamily was represented by five species and two genera (Table 2). One aspect to be noted is that the species from this subfamily found within our sampled catchment were mostly stenotopic and habitat specialists. *Chlaenius nitidulus* was the only more abundant species, subdominant in buffered and unbuffered habitats. The rest of the species, had consistently low relative abundances and frequencies, as compared to other carabid species, and were distributed in most site types, with the exceptions of *Chlaenius spoliatus,* found just in unbuffered sites and *Callistus lunatus*, found solely in one buffered site, as a singular occurrence.

The Nebriinae subfamiliy summed up three species, each from a different genera, all mostly encountered in densly vegetated sites. Of the three, *Nebria brevicollis* was by far the most numerous in all site types, ranking as the forth most abundant species in buffered sites. *Notiophilus palustris* followed as the second most abundant species of the subfamily, with presence observed solely in forested and buffered sites, alltogather with reduced relative abundances and frequencies. Lastly, the stenotopic species *Leistus piceus* was represented in just one buffered site by a lone individual, thus respresenting the rarest species of the group, and one of the rarest sampled within the entire catchment.

The Licininae subfamily was comprised of just two species, both of which with very reduced relative abundances and frequencies. *Licinus depressus* was found in few sites, in three of the four site types, with half of its total abundance at the most impacted sites, while *Badister bipustulatus* was found in just one buffered site in two instances (Table 2).

The Brachininae subfamily was represented by just one species, *Brachinus crepitans,* found to the highest degree in matrix sites, in I14 and to a much lesser extent in buffered sites, in R8 (Table 2, Figures 3 and 4). Subfamily Broscinae was also represented by a single species, *Broscus cephalotes*, found with reduced degree of representation, mainly in forest sites and sporadically in unbuffered sites (Figures 3 and 4). *Cicindela germanica* was the sole representative of the Cicindelinae subfamily though the middle catchment of the Argeș and it was found with very reduced relative abundance and frequency, solely in one unbuffered site (Table 2). The other singly represented subfamilies found during our survey consist of Panagaeinae and Scaritinae, represented by the rare *Panagaeus cruxmajor,* and, respectively, by the more common *Clivina fossor. P. cruxmajor* was found solely in one matrix site, with two singular occurrences. *C. fossor* was found to be relatively abundant in the matrix site where mostly present, yet a rare occurrence in the forest and unbuffered sites where it was sporadically found in low abundances. Lastly, the Lebiinae subfamily was found to be present in multiple site types and sites, however, mostly due to the state of the individuals, we were unable to identify them past genus.

The general patterns of distribution shown by carabid communities across the studied catchment and site types are generally in accord with tendencies reported in related literature. The higher percentage of carabid abundance in

epigean invertebrate communities in buffered and matrix sites may be linked to increased food availability. However, different mechanisms are involved in the two kinds of sites: in buffered sites it is the dominance of larger bodied caddisflies and longer emergence periods associated with better aquatic microhabitat conditions (Paetzold et al., 2006; Kupilas et al., 2021), whereas in matrix sites higher aquatic emerged biomass rates are mainly associated with small bodied chironomids favored by higher aquatic primary production (Kautza and Sullivan., 2016; Terui et al., 2018). More complex terrestrial microhabitat conditions generally associated with buffered riparian zones are also expected to increase both the species richness and abundance of ground beetles of such areas as opposed to similarly located habitats that lack arboreal vegetation (Kautza and Sullivan., 2016; Popescu et al., 2021). Within our catchment, buffered sites supported a higher number of rare species and pest predators, which, in accord with previous findings (Popescu et al., 2021), emphasizes the importance of riparian conservation in agricultural landscapes. The higher abundance of carabids within invertebrate communities and reduced number of species from matrix sites are likely attributed to two distinct factors. The reduced microhabitat habitat diversity of this site type supported, almost entirely, a few highly eurytopic and generalist agrobiont species in high numbers. Additionally, the availability of easily accessible prey from the nearby agriculturally converted patches may have facilitated the occurrence of epigean, highly mobile and voracious predators such as ground beetles (Batáry et al., 2007; Gallé et al., 2018). While unbuffered sites hosted some distinct open habitat specialists, it is generally shown by our findings and previous research (Batáry et al., 2007; Gallé et al., 2018) that riparian deforestation reduces the diversity and complexity of riparian carabids and the benefits they provide (Popescu et al., 2021; Stockan et al., 2014). Notably, the carabid communities in forest sites are the most distinct in the catchment, harboring more rare and vulnerable species. These findings highlight the importance of conserving headwater habitats to protect their unique biodiversity (Richardson and Danehy, 2007). The reduced abundance of carabids relative to the total of individuals sampled in forest sites can be explained by the increase of dead wood and associated physical habitat structures usually encountered in densely vegetated habitats and known to support other type of riparian predators such as web building spiders (Hunt et al., 2020; Ramberg et al., 2020). The widespread and high abundance of P. cupreus in Arges basin, including in unbuffered and matrix sites, aligns with previous findings reported in our county (Varvara et al., 2011) and internationally (Brygadyrenko, 2016; Porhajášová et al., 2014). The species is almost ubiquitous in agricultural landscapes, frequently reported in high abundances (Porhajášová et al., 2014; Varvara et al., 2011). Given their highly eurytopic nature and wide distribution, individuals from the species were used as model organisms in response to pressures associated with land use change, as well as agricultural practices (Sowa et al., 2022). Such research reinforced the idea that even the most resilient organisms can be significantly affected by anthropic activities, mainly through the reduction of individual fitness rather than changes in abundance which sometimes might be less significant. They highlight that functional traits might be better suited indicators than population size or community structure, at least in certain cases and reinforces the importance of complementary approaches in biodiversity research. It was shown that extensive monocultures, habitat reduction, pesticide use and other sources of pollution affect traits of *P. cupreus* individuals and their overall fitness (Marrec et al., 2017; Sowa et al., 2023). For instance, females of the species from crops intertwined with naturally occurring herbs and grasses have been documented to possess higher body mass and to have higher fecundity as opposed to ones from conventionally managed crops (Labruyere et al., 2016). Additionally, good practices such as sustainable crop management and promotion of landscape diversity were shown to positively influence the populations of *P. cupreus* and of other carabids (Labruyere et al., 2016; Marrec et al., 2017; Sowa et al., 2022).

In the middle catchment of the Argeș, Carabinae subfamily include several stenotopic species with very selective feeding preferences and specialized foraging straegies, recognised as

some of the most efficeint arthropod pest control predators (Ciubuc, 2021; Popescu et al., 2021). Species like *C. coriaceus, C. granulatus* and *C. caraboides* were fairly abundant in buffered sites, thus reinforcing the importance of such landscape structures to support varied populations of pest predators that are effective both numerically and functionally. Because the active periods of species vary throughout the year, more diverse communities are likely to sustain pest control over a longer duration. Increased species diversity may also facilitate a wider range of pest that can be prayed on by different carabids with different feeding preferences and degrees of specialization (Jaworski et al., 2023; Mitchell et al., 2014). The abundance of pest predators is also very important since the efficiency of the provided ecosystem service depends on the number of individuals actively hunting and on the quantity of prey consumed (Jaworski et al., 2023; Mitchell et al., 2014). Furthermore, the occurrence of protected (e.g., *C. variolosus*) and rare species (such as *C. sycophanta* and *C. intricatus*) indicates the relevance of forested and buffered riparian areas for supporting regional biodiversity within agricultural catchments. Among carabids and arthropods in general, *C. sycophanta* represents an archetype species for population control, as it was historically used to great effect against invasions of the larval stages of the gipsy moth (*Lymantria dispar*) and of other caterpillars, mostly within the United States (Weseloh, 1985). Aside from their rarity, species like *C. intricatus* represent valuable bioindicators used to assess the quality of woodland habitats since the presence of the species is generally correlated with old trees, as well as moist, cool and shady conditions (Pett et al., 2024). We argue this should emphasis not only the importance of buffer availability, but also of its characteristics, such as density and size, if such habitats are to effectively support rare and endangered species.

Platynus assimilis is one of the most interesting carabids sampled within the Middle Catchment of the Argeș River, given its clear dominance in buffered sites and relatively low abundances in other site types. The species is usually described as a stenotopic wetland specialist, though increasing reports of its presence in other nearby habitats, such as crops and meadows, tend to shift its perceived ecological preferences among researchers (Ploomi et al., 2012). It is clear, however, that individuals of this species can be used as biological control agents within agricultural landscapes, against a multitude of pest and with satisfactory effects (Kivimägi et. al., 2009). Furthermore, alongside the also dominant *P. cupreus*, *P. assimilis* represents perhaps one of the most studied ground beetles with regard to the effect of pesticide use on nontarget, beneficial taxa. Research on this topic commonly reported sublethal effects including prolonged hypoactivity (Tooming et al., 2014; Tooming et al., 2017), reduced pest consumption (Tooming et al., 2014; Tooming et al., 2017), reduced thermoregulation capacity (Kivimägi et. al., 2009; Merivee., 2015) and, increased respiration rate (Kivimägi et al., 2013). Evidence that less obvious traits and behaviors, such as shelter seeking, are affected by pesticides and reduce thermoregulation capacity highlights the need of incorporating trait-based approaches in studies of arthropods in agricultural catchments. In our context, *P. assimilis* is characteristic of buffered habitats and likely contributes to natural pest control due to its wide feeding spectrum and high abundances. As such, it serves as a clear example of the utilitarian value of forested riparian buffers in agricultural catchments. The low relative abundance and frequency of most other species, likely reflect normal community dynamics, where a few species, like *P. cupreus*, *P. assimilis,* dominate, while others are naturally less common. The sub dominance of *A. dorsalis* in matrix sites can be attributed to some evolutive adaptations of this species. Individual of *A. dorsalis* have been documented to be specialized aphid consumers, beneficial for pest control (Knapp, 2016; Kosewska and Nietupski, 2015) and perhaps the most resilient natural enemy with regard to agricultural land-use changes and pesticide use (Brygadyrenko et al., 2021). The species is extremely well adapted to starvation, with feeding gaps of individuals reported for as long as 218 days (Knapp, 2016). Individuals of the species seem to be extremely well adapted to avoid predation by implying a multitude of strategies, both morpho-physiologically such as aposematism (e.g., body odor defenses), as well as behavioral, such as fast hiding and aggregative strategies

(Brandmayer et al., 2006; Brygadyrenko et al., 2021; Knapp, 2016;). The species is one of the few among Carabidae that often aggregate, in its case, with individuals from genus Chlaenius, as also shown by our findings (Table 2 and Figure 4), strategy which increases their resistance to predators given the explosive defense strategy of Chlaenius (Brandmayer et al., 2006).

The more common occurring taxa from the Trechinae subfamily include individuals mostly associated with riverine habitats, such as Bembidion, Tachys and Ocys (Turin, 2000). Due to the reduced size of these carabids, it may be possible for the subfamily be underrepresented in our samples. The high frequency and abundance of *B. lampros,* preponderantly within matrix sites, is consistent with reports from literature which refer the species as mostly an open habitat specialist, often abundant in agricultural patches, feeding on aphids and lepidopteran larva (Mitchell, 1963). The species has been documented to exhibit a higher-than-average resistance to starvation (Petersen, 1999), a common characteristic reported for dominant species in matrix sites. *T. carpathicus* and *T. pilisensis* were sparse in the catchment, with the first also being endemic to the Romanian and Ukrainian Carpathian Range. Both species are strongly associated with old forests, humid conditions (Ciubuc, 2021) and have been encountered solely at higher altitudes.

By the contrary, *H. griseus* and *H. rufipes* are two of the most common and abundant species found in open habitats of agricultural landscapes (Farinós et al., 2008; Matalin, 1997; Tallósi et al., 2008). Both members of the Harpalinae subfamily are often found together, with one usually dominant and the other subdominant (Andrici et al., 2015; Farinós et al., 2008; Talmaciu et al., 2016; Talmaciu and Talmaciu, 2005; Varvara et al. 2001). Research results are divided regarding the dominance of one over the other and to the cause of such responses. Most findings seem to indicate that ratios between the two species fluctuate in response to habitat particularities, landscape characteristics, as well as seasonal and annual weather conditions (Farinós et al., 2008). Both species are known to be omnivorous and have a long history as predators of invertebrate pest, mainly aphids, slugs and flies (Farinós et al., 2008; Luff, 1980; Monzó et al., 2011), and, to a lesser extent, contribute to weed control by consuming seeds (Gaba et al., 2019; Harrison and Gallandt, 2012). However, some reports of crop damage also exist (Gîdei and Popescu, 2012). Generally, it is considered that *H. grisus* is less common than its sibling (Ciubuc, 2021), as some sources classify it as stenotopic and more attracted to less disturbed habitats within agricultural landscapes (Desender and Turin, 1989; Tallósi et al., 2008). This is also the case in our catchment since the species is dominant and have the highest abundance in buffered sites. Of the two, *H. rufipes* is undoubtedly the most studied, as research of its potential for pest control (Luff, 1980) and response to insecticides is around half a century old (van Dinther, 1963) and since individuals of the species were extensively observed in multiple studies related to carabid migration (Matalin, 1992) and bioindication (Allegro & Sciaky, 2003; Sipos et al., 2024). The other species of the subfamily were much less abundant and widespread, however, equally of interest from scientific and conservation standpoints. *H. affinis* is an adaptable, polyphagous species, primarily associated with open and dry agrarian habitats, and has been extensively studied for its broad-spectrum weed seed predation. *S. teutonus* and *D. germanus* were among the rarest occurring species we identified. However, *S. teutonus* is an agrobiont species used as a bioindicator of crop habitats quality, found in spring in organic, biodynamic agroecosystems, or set-aside field for the purpose of regional biodiversity. Though documented to exhibit a strong affinity for open and sparsely vegetated habitats, it was also documented in riverine habitats and even in the presence of arboreal vegetation since it may be attracted by supplemental food sources available in edge habitats (Turin, 2000). The presence of this species in buffer sites support its bioindicator value within agricultural catchments. The presence of *D. germanus* in the matrix sites, can be indicative of lack or reduced use of pesticide. Historically abundant in crops and open habitats, the species significantly declined within its distribution range to the point where it has become endangered and rare in its European habitats (Müller-Motzfeld, 2023). The increased use of pesticides and other intensive practices, such as lack of reserve patches,

riparian included, or intensive tillage represent some of the main drivers of biodiversity decline within agricultural landscapes. Preservation of riparian buffers should be prioritized among agricultural catchments as one of the most effective means of producing more sustainable crops and food for the human population. *Chlaenius nitidulus* is a riparian specialist frequently associated with moist and open habitats (Stančić, 2010; Turin, 2000), features also supported by our findings. As most species from its subfamily to be found within our studied catchment, it is regarded as stenotopic and fairly rare among carabid communities in general (Boscaini et al., 2000; Kędzior et al., 2016). *Chlaenius spoliatus,* also associated with riparian habitats, has been reported as relatively frequent, but typically occurs in low abundances within wetland carabid communities (Boscaini et al., 2000; Cardarelli & Bogliani, 2014). Its scarcity appears to be a species-specific trait, and with other confamilial taxa, highlights the potential importance of non-forested riparian habitats in supporting specialized predators along riverine corridors. *C. lunatus* represents the rarest species of its subfamily found within our study catchment and one of the rarest of Chlaeniinae in general, however not protected. It is an open habitat specialist, usually found in xerophilous meadows and places with chalky soils (Gîdei & Popescu, 2012).

Licinus depressus is a stenotopic, xerophilous species, known to prefer chalky soils and generally associated with open, as well as, sparsely or mildly vegetated habitats (Brygadyrenko, 2015). Individual of the species are well documented feeding specialists, known to voraciously attack and consume land snails (Baur et al., 2023). Though their hunting strategy and feeding preference have been thoroughly addressed, we were unable to find any research where the species has been clearly referenced or used as a biological control agent. We argue this may be due to the generally reduced abundances of *L. depressus* reported throughout various habitats (Brygadyrenko, 2015), also recorded in our research area. Contrarily, *B. bipustulatus* is a highly eurytopic and hygrophilous species, relying extensively on scavenging and widely used as indicator of land use change, especially of urbanization (Brygadyrenko, 2015). The species has been used as a pest predator and is seemingly more resistant than other carabids to the effect of pesticides (Brygadyrenko, 2015). In our agriculturally dominated catchment, this species is rare and sporadic, likely as a result of multiple interacting factors, including the absence of urban habitats in the immediate vicinity of the sampling sites and competition with better adapted species within the areas sampled.

The prevalence of the bombardier beetle, *B. crepitans*, in agricultural habitats aligns with previous reports (Brigić et al., 2009; Fiera et al., 2013; Varvara, 2016). While the species shows a preference for dry, open habitats, it is not restricted to them, as it has also been recorded in forested habitats (Varvara and Șerban, 2002). Notably, this is the only species we found that develops as larval ectoparasite of other carabids, mainly of the genus *Amara,* with which it commonly co-occurs (Saska & Honek, 2004; Saska & Honek, 2008), a pattern also noticed in our samples. The species does not pose conservation concerns, yet some sources mention its potential for crop pest control either during larval and adult stages (Saska & Honek, 2004).

The distribution and abundance of *B. cephalotes* within the middle catchment of Argeș are somewhat unexpected given the usual preference of this stenotopic species for relatively dry, open habitats and to a lesser extent for densely vegetated habitats (Matalin & Budilov, 2003). Generally, it usually occurred as subdominant in their respective communities (Aleksandrowicz et al., 2009; Lövei, 1984; Sorokin, 1981). Especially in unbuffered habitats, its presence can be reduced by competition with often co-occurring, eurytopic and more voracious species such *as P. cupreus* (Kabacik-Wasylik, 1971). Its use as a pest control agent may be hindered by its reduced effectives and high plasticity in feeding behavior (Mossakowski, 2003). However, multivoltine development of the species (Matalin & Budilov, 2003) might provide an advantage to pest predation (Kabacik-Wasylik, 1971; Mossakowski, 2003) when competition is lower. We found only a few individuals of the Lebiinae subfamily, all in poor condition and unidentifiable to species level. Individuals of the genus *Syntomus* are typically small and usually very mobile (Turin, 2000). Due to the taxonomic

uncertainty and their overall reduced abundance, we cannot asses their significance to our research. However, it is worth noting that all the documented European species are carnivorous (Turin, 2000) and in our case, they were most abundant and widely distributed in buffered and forest sites. This pattern may further support the role of such riparian habitats in enhancing the diversity and effectiveness of natural pest control.

CONCLUSIONS

Our main findings align with the central tendencies of previous research, with increased diversity of stenotopic, rare and protected species recorded in buffered riparian habitats. In addition to their contribution to the overall species diversity within the catchment, despite the lower relative abundances, buffered riparian zones supported a more diversified array of specialized predators known as some of the most efficient crop pest predators.The occasional or accidental occurrence of certain vulnerable species, many with limited geographic ranges, highlights the urgent need for conservation efforts not only along the headwaters and downstream riparian zones, but also in adjacent habitats where biodiversity regulations have historically been scarce. Sustainable practices, such as promoting biological control through natural pest predators and preserving of buffered riparian habitats, should be widely adopted in agricultural landscapes, where both crops and biodiversity face growing threats from pollution, invasive species and other anthropogenic pressures.

Other landscape features, such as set aside fields or, at least, patches spared of intensive agriculture, can also contribute significantly to overall landscape diversity.

Future research on carabid communities within the Middle Argeș catchment should include a detailed analysis of functional traits and diversity metrics, while also prioritizing the identification of key environmental variables that shape their dynamics. This approach is essential for advancing our understanding of their ecological roles and for emphasizing the importance of riparian zone conservation as a sustainable land management practice.

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THE IMPORTANCE OF COMMERCIAL FISHERIES IN THE ROMANIAN PART OF THE DANUBE RIVER, EVOLUTION AND CHALLENGES

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Abstract

In Romania, activities related to fishing, aquaculture, processing and trade in fish and fish products are widespread in all regions of the country. In some remote areas, such as the Danube Delta and Danube Plain, and the Danube Cluster, fishing is one of the main activities, providing jobs, income and food for the local population. In the context of increasing environmental and economic pressures on the Danube River, it is essential to adopt integrated measures to protect biodiversity and secure water and fish resources for local communities. While Romania ranked second in Europe in 2011 for inland fisheries according to the Institute for European Environmental Policy, by 2023 it had fallen to 17th place, as indicated by Eurostat data, reflecting significant changes in the sector. Given these considerations, the paper aims to present an analysis of the evolution of commercial fishing in the Romanian part of the Danube River in relation to other riparian countries and to highlight the challenges of this activity, challenges that can serve as the basis for a sustainable management strategy.

Key words*: effective public policies, fish management strategy, sustainability of aquatic ecosystems.*

INTRODUCTION

According to Law No. 176/2024 on Fisheries and the Protection of Living Aquatic Resources, commercial fishing is defined as "*Activities of extracting/harvesting living aquatic resources from natural aquatic habitats for commercial purposes*". In other words, commercial fishing, both freshwater and marine, is an important sector both economically and socially, playing an important role in ensuring food security and livelihoods for many communities (Sumaila et al., 2014; Pila et al., 2023), as fish constitutes one of the major sources of proteins (Holmlund & Hammer, 1999; Samofatova & Neveseliuk, 2024).

In 2022, the freshwater fishing industry registered a total capture of 695.398.700 TLW (tons live weight) globally (FAO, 2024), fully influenced by the growth of the global population and human fish consumption.

In Romania, this activity has a long-standing tradition, particularly in regions with access to rivers and lakes, where fish resources have been exploited over time for domestic consumption and trade. The Danube River, as the country's main hydrographic basin covering an area of 801,463 km² and hosting a population of 79 million people from 19 countries, plays a central role in the fishing industry, providing a diverse habitat for numerous commercially significant fish species.

However, over the past decades, the global commercial fishing industry, a multibillion-dollar sector operating on a multinational scale, has significantly contributed to the depletion of fish populations, pushing numerous species toward the brink of extinction. Scientists estimate that between 0.97 and 1.97 trillion wild fish are caught and killed annually worldwide (Wickens, 2023), further straining fragile aquatic ecosystems.

Challenges such as pollution from oil spills, agricultural runoff, vast amounts of waste, invasive species (Wong et al., 2007), overfishing, also degraded both marine and

freshwater habitats, exacerbating the challenges faced by fisheries (Winiwarter et al., 2013).

These global issues have been reflected in Romania's commercial fishing sector as well, particularly in the Danube, where fish stocks are declining due to IUU fishing (Illegal, Unreported and Unregulated), overfishing and environmental degradation (Kalikoski et al., 2018). This has placed significant pressure on local fishing communities, threatening their livelihoods and the economic sustainability of the sector and the river ecosystem. The decline in fish stocks in the Danube, caused by both overexploitation and ecosystem changes, has led to a significant decrease in catches, affecting the sector's economic sustainability. Additionally, competition with imported fish products and changes in fish demand have negatively impacted the local market, limiting development opportunities for traditional fishers (Neculita and Moga, 2015; Hodosan et al., 2023).

Beyond economic and environmental pressures, the sustainable management of commercial fishing in Romania also involves challenges related to public policies and regulatory enforcement. Discrepancies between national and European legislation, difficulties in implementing conservation measures, and the lack of integrated support strategies for fishing communities are aspects that require a comprehensive approach, and solutions tailored to local realities.

This paper aims to analyse the evolution of commercial fishing in the Romanian sector of the Danube, highlighting legislative factors on this economic activity. Furthermore, trends in Romania will be compared with those in other riparian countries to better understand the sector's position and prospects at a regional level. Finally, potential strategies for the sustainable management of fish resources will be discussed to ensure both biodiversity protection and the continuity of economic activities based on fishing.

MATERIALS AND METHODS

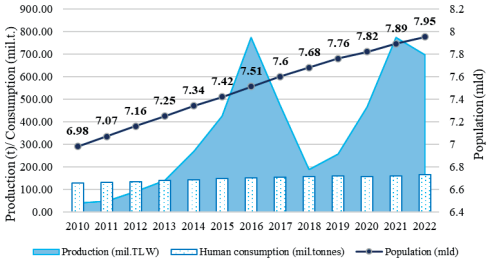
To identify the trend in the commercial freshwater fishing sector, data was processed from the Software for Fishery and Aquaculture Time-Series Statistics (FishStatJ_4.04.00) of the Food and Agriculture Organization of the United Nations (FAO), the Eurostat database, as well as commercial fishing data from the National Agency for Fisheries and Aquaculture (www.anpa.ro). Additionally, official data from the National Institute of Statistics (www.insse.ro), and the Federal Reserve Bank of St. Louis (www.stlouisfed.org) were used.

Furthermore, Romanian fisheries legislation was analyzed to identify strengths and weaknesses in this sector, providing a comprehensive perspective on the regulatory framework.

The data was statistically processed for normality using the Shapiro-Wilk Test (95% confidence interval). Correlations were determined by calculating the Pearson correlation coefficient.

RESULTS AND DISCUSSIONS

The fishing sector is vast and complex, based on many species, making the analysis of trends more difficult (Sumaila et al., 2014).



N.B. The *Production* Indicator only takes into account fish catch, excluding crustaceans, molluscs, and algae; The *Human Consumption* Indicator does not include imports, exports, stock variations, or non-use products.

Figure 1. Evolution of commercial fishing worldwide, 2010-2022 (Source: original)

Between 2010 and 2022 (Figure 1), global fish capture exhibited an oscillatory trend, with peak values recorded in 2016 (774.08 million TLW) and 2021 (772.29 million TLW). The lowest capture volume was observed in 2010, at 39.59 million TLW. This trend can be largely attributed to demographic growth (correlation coefficient: 0.742), increased consumer demand (correlation coefficient: 0.743), and rising global fish product prices. The Harmonized Index of Consumer Prices for Fish and Seafood (2015 = 100) increased from

88.82 in 2010 to 126.36 in 2022, reflecting a significant price escalation over the analysed period. These factors have strongly stimulated production in order to meet the growing market demands, driving a dynamic market response to the growing global demand for fish and fish-based products (Dobre et al., 2024).

Romania, a riparian country with a rich history in commercial fishing, has linked this activity to various historical periods, dating back to the Neolithic era (Tatulici, 2020), the Ottoman period, when fishing was administered through a 15% In-kind lease system (Belacurencu, 2006), and up to the first Romanian Fishing Law, introduced by Antipa in 1896, as a response to the sector's growing activity (Bulat et al., 2023). At that time, the fishery resources covered 700,000 hectares, with the Danube River being the primary fishing basin. The Danube Delta alone encompassed approximately 1,616 km² of natural aquatic basins, where fish stocks were highly sought after (Belacurencu, 2006).

Currently, Romania's hydrographic network spans 843,710 hectares, accounting for over 3.5% of the country's total surface area. Public water bodies include: 300,000 hectares of natural lakes and ponds, 98,000 hectares of reservoirs and polders, 47,000 km of lowland and hilly rivers, 19,000 km of mountain rivers, and 1,373 km of the Danube River.

According to Stanciu et al. (2015), Romania's fisheries sector experienced a significant decline between 1989 and 2015, primarily due to the depletion of domestic resources and the disappearance of the Romanian oceanic fishing fleet - a challenge that remains unresolved to this day.

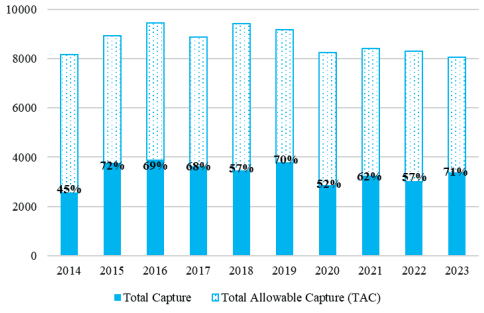


Figure 2. Evolution of the commercial fishing sector between 2014-2023 (Source: original)

Commercial fishing is one of the main sources of income for Romania, particularly in delta communities. In this context, total catches have seen a significant increase, from 2,540.9 tons in 2014 to 3,354.1 tons in 2023 (Figure 2). However, the demand for fish and fish products has not evolved as sharply, with per capita consumption rising modestly from 4.9 kg in 2014 to 6.5 kg in 2023, reflecting an average annual growth of only 3%, unlike in Ukraine, where the annual per capita fish consumption averages 13.2 kg (Samofatova & Neveseliuk, 2024).

This increase in demand, coupled with declining catches and new trade policies, has also affected the average price of fish products, which rose from 104.9 lei in 2014 to a peak of 133.92 lei in 2022 (Figure 3), reflecting an inflationary trend and market volatility. In other words, as consumption increases, catches should also grow at the same rate in order to control price increases. However, in 2023, the price experienced a sharp drop to 103.33 lei, suggesting a slight market stabilization, but not enough.

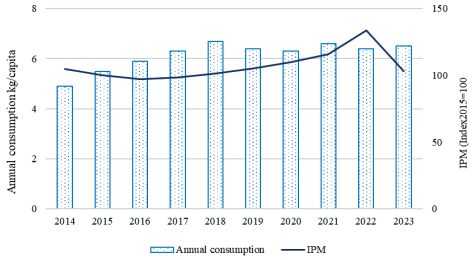


Figure 3. Evolution of market trends of the Romanian fishing sector 2014-2023 (Source: original)

However, given that the actual catch represents, on average, only 63% of the Allowable Catch, there is a significant untapped potential in the commercial fishing sector. This discrepancy may indicate either restrictions imposed to protect aquatic resources or logistical and economic challenges that prevent the full exploitation of available quotas, or unreported catches. Unreported catches or IUU fishing represent one of the biggest threats to fish stocks and have severe implications for the sustainability of the sector.

Additionally, considering that approximately 114,651 tons of fish and fish products are

imported annually, according to the FishStatJ platform, the market's dependence on imports to meet the demand not covered by domestic production can influence price fluctuations and industry stability, leading to increased economic vulnerability and potential market volatility depending on external factors such as currency fluctuations or changes in international trade policies. This situation was also highlighted by Stanciu et al. (2015). Moreover, according to Dima et al. (2021), the human demand for aquatic products will increase to 40 kg/capita until 2040. The issue is also relevant in Bulgaria (Todorov, 2020) and Ukraine (Samofatova & Neveseliuk, 2024), where high demand for fish and fish products has led to increased imports from the European community.

Commercial fishing, as defined by Law No. 176/2024 on Fisheries and the Protection of Living Aquatic Resources, refers to the exploitation of living aquatic resources from natural fishery habitats for economic purposes. On the Danube, this activity is carried out by professional fishermen and specialized companies using specific techniques and equipment for capturing and marketing fish. Strictly regulated by authorities, commercial fishing requires special licenses and permits to be legally practiced.

Fishery policy in Romania has evolved significantly over the past few decades, addressing issues related to the conservation of fish resources, the development of aquaculture, and the promotion of sustainable commercial fishing. However, there are challenges, and collaboration between authorities, fishermen, local communities and the private sector is essential to achieving the sustainable development goals of the fisheries sector.

Fisheries policy is a fundamental instrument in the sustainable management of aquatic resources, playing a direct role in biodiversity conservation and the economic development of the fishing sector. Through well-defined strategies and effective measures, this policy aims to maintain a balance between resource exploitation and ecosystem protection, ensuring long-term sustainability. Fisheries policy in Romania aligns with the Common Fisheries Policy (CFP), the legislative and regulatory framework adopted at the European Union level. Despite facing significant challenges, the CFP plays a crucial role in ensuring the responsible and sustainable use of aquatic resources in Europe.

Usually, the European Union sets the main framework through its policies, leaving each country the freedom to implement them according to their specific conditions. This happens because each country has its own economic, social, and ecological circumstances, which can influence how European regulations and directives are applied. For instance, in the field of fisheries and aquaculture, while the European Union adopts common strategies for managing aquatic resources and protecting biodiversity, each member state is responsible for adapting these policies to its own needs and particularities.

This flexibility allows countries to consider factors such as the freshwater species in their areas, specific climatic conditions, the structure of the domestic market, and cultural traditions related to fishing and aquaculture. It also enables national authorities to set specific measures to address local challenges, such as protecting certain aquatic habitats, reducing pollution, or supporting sustainable fishing. This process allows for a more nuanced and adaptable approach to managing aquatic resources and the fishing industry, contributing to the long-term sustainability of this essential sector.

Due to its geographical position and extensive network of rivers, lakes, Romania has significant fisheries potential. Its diverse ichthyofauna and rich aquatic ecosystems have led to the development of a fisheries policy focused on three main objectives: conserving and protecting fish species, stimulating fish production, and responsibly managing commercial fishing and aquaculture. Through current regulations, Romania seeks to minimize negative environmental impacts while supporting the economic development of communities dependent on this sector.

However, in recent years, fishery resources have been severely affected by numerous factors, leading to a decline in fish stocks and threatening the sustainability of commercial fishing. Climate change is one of the most significant threats to aquatic ecosystems, negatively impacting habitat quality and the

reproductive capacity of many species. Rising global temperatures affect water productivity, reducing food sources for fish and disrupting natural reproductive cycles. Additionally, climate change leads to decreased river flow, the reduction of wetland areas, and alterations in water chemistry, potentially causing species extinction and major imbalances in the aquatic food chain.

Another significant factor contributing to the degradation of fishery resources is water pollution, caused by industrial, agricultural, and urban activities. The discharge of toxic substances, excessive use of pesticides and fertilizers in agriculture, and the accumulation of household waste in rivers and lakes degrade water quality and endanger fish survival. Pollution with heavy metals and microplastics, increasingly present in freshwater and marine environments, can have devastating effects on ichthyofauna and, consequently, on the fishing industry. According to Raykov (2020), this situation is generally valid, not only for the Romanian waters, but also for the small-scale fisheries in Bulgaria.

Moreover, overexploitation through excessive fishing and poaching worsens the situation, drastically reducing fish populations and disrupting aquatic ecosystems. In many areas, catches exceed the natural regeneration capacity of fish populations, leading to severe declines in certain species and even their disappearance from specific habitats. The lack of strict monitoring and enforcement of responsible fishing regulations allows destructive practices to continue, harming both the environment and the economic sustainability of the fisheries sector.

Economic difficulties faced by commercial fishers further challenge the viability of the fishing industry. High costs of equipment, infrastructure, and fuel, combined with market fluctuations and competition from imported products, create major obstacles for local producers. In many cases, fishers are forced to scale down or even abandon their activities, leading to decreased production and a reduced contribution of the fisheries sector to the national economy.

The protection of fishery heritage involves a balanced management and sustainable administration of fishing activity, considering the aquatic resources as main element.

Authorities responsible for fisheries management have the duty to formulate the Total Allowable Catch Order (Order no. 75/874/2024) to monitor and enforce established catch limits for each commercial specie, imposing strict controls on fishing activities to ensure compliance. In contrast, the Bulgarian regulation for the TAC is set only for a few species from the Black Sea (Raykov, 2020).

Also, regulations regarding the ages of fish specimens are set and fishermen are required to report the quantities of fish caught and to adhere to regulations aimed at protecting and conserving fishery resources, thus ensuring their sustainable use. These limits are set based on species, geographical areas, and specific time periods, and are expressed either in terms of weight (tonnage) or the number of fish and ensure that will not be captured before reaching sexual maturity and reproduction. Typically, these limits are based on scientific studies and stock assessments to determine the number of fish that can be harvested without compromising the viability and regeneration of fish populations.

Additionally, authorities establish annual closed seasons and areas where fishing is partially or completely prohibited, to allow for the reproduction and regeneration of fish populations, thus contributing to the conservation of aquatic habitats and biodiversity. For example, the sturgeons are totally prohibited to be commercially fished in the Romanian sector of the Danube River, considering their status as an endangered species. Poaching and illegal sturgeon trafficking in the Lower Danube region pose a major threat to the survival of endangered sturgeon species. Their capture for meat and roe, highly valuable products on the black market, fuels an illegal circuit that contributes to the rapid decline of their populations. This situation jeopardizes not only the biodiversity of the region but also the aquatic ecosystems that depend on the presence of sturgeons, as they play an essential role in maintaining the ecological balance of the Danube. Furthermore, for local communities, poaching represents an economic risk, as the fishery resources in this

area are vital for sustainable fishing (WWF Romania, 2024).

To support the sustainable development of the fisheries sector, public policies must adapt to new realities by ensuring financial support for the development of the fishing sector.

In the context presented above, Table 1 highlights the main strengths, weaknesses, opportunities, and threats affecting the commercial fishing sector in Romania, reflecting both the challenges, such as climate change and pollution, as well as the opportunities for development and sustainability of the industry, in line with the existing legislative framework and policies.

Table 1. SWOT Analysis of the commercial fishing sector in Romania

STRENGHTS
Existence of fishery resources at an industrially exploitable level
Local knowledge and experience - Traditional fishermen have a deep understanding of habitats and species behaviour.
High biological diversity - Presence of valuable species and favourable habitats for various fish species
Tradition of fish consumption
High demand for aquatic products
Advantageous geographical position in relation to European markets
Existing legal framework for resource protection - There are regulations for the management and protection of fish stocks.
WEAKNESSES
Lack of accurate information on stock status
Absence of scientific research programs in the fisheries sector
Out-dated fishing boats, engines, and equipment
Poor cooperation between fishermen and authorities - Lack of effective dialogue between industry stakeholders and decision-makers
Dependence on weather conditions - Seasonal variations affect production and fish availability on the market
Poorly developed infrastructure
Traceability and certification issues - Difficulties in monitoring catches and implementing quality standards
Lack of incentives for young people - Aging workforce in the fisheries sector and low interest from new generations
Strict fishing regulations
High dependence on subsidies - Fishing is often reliant on financial support to remain profitable.
Low level of digitalization - Administrative procedures and catch monitoring are not sufficiently automated.
Lack of an efficient system for promoting fish products - Local products do not benefit from adequate marketing to compete on the international market.
OPPORTUNITIES
Large potential for absorption in the domestic market and expansion of export markets - Increasing demand for fish and fish products in international markets.
Promotion of traditional products and eco-certification (e.g., 'local product' or 'sustainable fishing') - Adding value through branding and certification.
Growing interest in healthy foods - Global trends toward healthy eating can boost demand for aquatic products.
Development of short supply chains - Direct sales from producer to consumer can increase fishermen's incomes.
Adapting to climate change through sustainable policies - Plans for resource protection and reducing environmental impact.
Investment in Research and Innovation - Funding for developing and implementing new, sustainable technologies and practices to increase efficiency, productivity, and sustainability
New technologies for stock monitoring - Use of drones, GPS, and artificial intelligence for more efficient management
Potential for developing related activities such as ecotourism and educational programs on environmental conservation.
Implementation of advanced modern technologies - Attracting younger generations to the sector
Access to European funds for modernization - Financing available for infrastructure, equipment, and sustainability initiatives
Development of sustainable aquaculture - Possibility of compensating for declining catches through fish farms
Protection and restoration of fish habitats - Environmental projects aimed at rehabilitating fish breeding areas
Digitalization of fishing activities - Implementation of online licensing and reporting systems
THREATS
Overexploitation of resources and poaching
Impact of climate change - Rising temperatures and changes in ecosystems, decreased water levels in rivers and lakes can negatively affect fish populations and the spread of diseases, while drought events can impact fish habitats and access to resources.

Invasive species - The spread of foreign species can disrupt ecosystem balance and affect valuable native species.
Increased competition from industrial aquaculture - Large fish farms may reduce demand for wild-caught fish.
Fishing bans or restrictions
Rising production costs
Difficult access to and bureaucratic procedures for obtaining fishing funds
Limited administrative capacity and excessive bureaucracy
Legislative instability
Pollution - Water pollution pose serious threats
Economic instability and price fluctuations - High fuel and equipment costs can affect the profitability of fishing.

Given the existing strengths of the industry (Table 1), such as high biological diversity and a strong tradition of fish consumption, it is important to capitalize on opportunities like expanding export markets, investing in research and innovation.

However, addressing the weaknesses, including outdated infrastructure, lack of digitalization, and poor cooperation between stakeholders, requires targeted policy interventions and modernization efforts.

Moreover, organizing the fishing sector, implementing concrete measures to protect aquatic resources, strengthening monitoring and control systems for fishing activities (Order 1369/2018), developing effective fish restocking programs with native species, reducing pollution, and promoting sustainable fishing practices are among the necessary solutions to secure the sector's future. These initiatives are particularly important in mitigating threats such as overexploitation, climate change.

Additionally, investments in education and professional training for fishers, as well as financial support during fishing bans, could contribute to enhancing the sustainability of commercial fishing in Romania. Encouraging young generations to enter the sector by providing incentives and modernizing fishing techniques would help counteract the aging workforce and declining interest in this profession.

Through marketing campaigns and supporting the establishment of direct sales platforms, the government and organizations in the fishing sector should promote the valorisation of Romanian fish products on both domestic and international markets, to help commercial fishermen access new markets and obtain better prices for their products. In 2019, the fish exchange in Tulcea was opened for the auction and commercialization of fish products obtained from the Danube Delta, with the aim of creating a competitive and functional market. However, at present, the platform is no longer operational (MADR, 2019), highlighting the need for sustained efforts to ensure its long-term success.

Furthermore, adapting fishing regulations according to the reproductive cycles of species, as well as considering geographical differences and target species, is essential for effective resource management (Order no. 23/297/2025). Aligning these regulations with EU policies and sustainable fishing practices will ensure a balance between economic viability and environmental conservation, securing the future of Romania's fisheries sector.

CONCLUSIONS

The commercial fishing sector in Romania benefits from rich natural resources. However, overfishing and water pollution pose major risks that threaten the sustainability of these resources.

Although Romania has significant potential for fish product production, there is a continued reliance on imports to meet domestic demands. This highlights the need to develop more efficient aquaculture methods and stimulate domestic production.

Climate change represents a significant threat to aquatic habitats and fish biodiversity. Rising water temperatures and changes in hydrological regimes can negatively affect fish stocks, and the fishing sector must adapt quickly to address these changes.

Poaching remains a serious problem in Romania's commercial fishing sector. Stricter control of fishing activities and increased sanctions can reduce this issue, helping to protect natural resources.

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