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GENETICS AND BREEDING

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EFFECT OF NON-GENETIC FACTORS ON BIRTH WEIGHT IN A POPULATION OF TELEORMAN BLACK HEAD SHEEP BREED

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Abstract

Considering the increasing demand for sheep meat, it is necessary to optimize the breeding strategies and the management of sheep breeding in Romania in order to satisfy this demand by obtaining animals with high genetic potential in terms of body development traits. Knowing that some of the main factors such as sex, parity, type of birth are considered sources of variation of several growth-related traits within the sheep species, the aim of the present work was to determine the influence of these factors on the birth weight of a population of Teleorman Black Head sheep. In order to achieve the scope, the birth weight character of 732 lambs born in the 2021 lambing season was studied. The data obtained by weighing were statistically processed in Excel using the software `Real statistics`. The sex ratio was 1:0.77, with 407 females and 316 males, which had an average birth weight of 4.053 ± 0.039 kg and 4.19 ± 0.044 kg, respectively. The results showed that birth weight was insignificantly influenced by sex (p>0.01), strongly influenced by parity (p<0.01) and strongly influenced by birth type (p<0.01).

***Key words**: birth weight, growth traits, non-genetic factors, Teleorman Black Head breed.*

INTRODUCTION

Birth weight is a critical factor that affects the survival and growth rate of lambs. Non-genetic factors such as breed, sex, parity, litter size, and environmental conditions have been shown to influence birth weight (Thiruvenkadan et al., 2008; Assan and Makuza, 2005; Vlahek et al., 2021; Assan, 2013; Popa et al., 2020; Assan, 2020). Understanding the impact of these factors is vital in developing effective management strategies to enhance lamb productivity.
Previous studies have highlighted the significant effect of sex, type of birth, and season of birth on birth weight (Thiruvenkadan et al., 2008). Additionally, maternal nutrition, age, and body condition were found to significantly influence birth weight (Assan, 2013). Other studies have shown that litter size and the presence of stressors during gestation affect birth weight (Vlahek et al., 2021; Assan, 2020). Furthermore, genetic factors have been shown to play a role in determining birth weight (Popa et al., 2020).
Given the importance of birth weight on lamb productivity, this paper aims to investigate the impact of non-genetic factors on birth weight in a population of Teleorman Black Head sheep breed.
The genetically determined variations in the birth weight of lambs are most often associated with differences between breeds. The existence of within breed variations in birth weight supports the fact that nongenetic factors also influence this trait. Therefore, adjustment for non-genetic factors contributes to a more accurate estimation of birth weight (Mahala et al., 2019).
Teleorman Black Head sheep is a breed with early sexual maturity, moderate-high daily gain (Grosu et al., 2005 cited by Pelmuș et al., 2019) and which has started to be increasingly bred in large numbers due to its massive body shape and higher body weight, as well as its increased capacity in milk production compared to other native breeds from Romania (Tsurcana and Tsigai).

MATERIALS AND METHODS

The study was conducted on a number of 732 lambs born in the 2021 lambing season of 657 ewes from Teleorman Black Head sheep breed.

Ewes are included in the Official Performance and Recording Scheme (C.O.P), which implies compliance with a set of rules related to the period and method of breeding and the management of rearing. Therefore, the breeding season began in early September and ended in mid-October, with one ram randomly assigned to 30 ewes for breeding, while strictly avoiding inbreeding. Lambing occurred in the months of February and March, with the ewes separated from the rams. Regarding the feeding system, the sheep were grazed throughout the warm season and were given a concentrate feed made from a mixture of maize and sunflower cob. During the cold season, the sheep were fed with high-quality hay, the concentrate feed made from a mixture of maize and sunflower cob, and a vitamin-mineral premix supplement. The birth weight (BW) of lambs was determined immediately after birth using an electronic scale. Data related to parity, type of parturition, and sex of the offspring were collected on-site, from the reproductive records of the farm and from the electronic platform of the genealogical register of the Teleorman Black Head sheep breed (https://rg.registrulgenealogic.ro).

Categorical variables (considered as non-genetic factors) (Vlahek et al., 2021), were defined as follows: sex (male - female), parity (1-5), birth type (single - twin).

For statistical analysis, multiple linear regression analysis was used to examine the influence of sex, parity and lambing type and the fixed effects model used which describes this influence was:

$$Y_{ijklmn} = \mu + S_i + P_j + T_k + e_{ijklmn}$$

Where:

Y_{ijklmn} = analized trait (BW) birth weight;

μ = overall population mean;

S_i = fixed effect of i^{th} sex of lamb;

P_j = fixed effect of j^{th} parity;

T_k = fixed effect of k^{th} lambing type;

e_{ijklmn} = residual error.

To test if there are significant influence of fixed factors on birth weight, the analysis of variance (ANOVA) was used followed by a Tukey HSD test for highlighting the significance of differences between each individual groups within the same fixed factor. The single factor ANOVA as well as the descriptive statistics was applied for all groups of lambs from each of the non-genetic factor studied. All results were tested at the significance level of P<0.01.

The data analysis for this paper was generated using the Real Statistics Resource Pack software (Release 7.6), Copyright (2013-2022) Charles Zaiontz.

RESULTS AND DISCUSSIONS

The sex ratio among the total lambs born was 1:0.77 (i.e. 407 females and 316 males). The results showed that the average BW for the whole population of lambs examined was 4.11 ± 0.79 kg, values that are close to those found by Ptáček et al. (2017) who obtained at a population of Suffolk lambs an average BW of 4.8 kg and also for a population of Île-de France breed lambs, Ivanova et al. (2017) obtained an average BW of 4.53 ± 0.042 kg. These values could predict a high daily gain for lambs of Teleorman Black Head sheep and also that this breed has good meat production performance, similar to those of breeds specialized for this production.

Table 1. Descriptive statistics for the birth weight of Teleorman Black Head lambs

Var.	N	$\bar{X}$	SD	V%	Min.	Max.
BW (kg)	723	4.11	0.79	19.33	2	5.8

Regarding the differences in BW between groups, significant ones (P<0.01) has been recorded within two of the fixed factors: parity and type of lambing. We oberved that BW increased with the increase in parity (Figure 1), so lambs from the first parity had an average weight of 3.83 ± 0.05 kg and lambs from the fifth parity 4.22 ± 0.09 kg (Table 2). The same observation that highlights the upward trend of body weight with increasing parity has been reported by other authors as well on different sheep breeds Murphy et al. (2020), Gootwine and Rozov (2006), and Vlahek et al. (2021). The average BW of lambs from first parity ewes was significantly different from the average BW of lambs from the fourth parity (p<0.01). Same differences was recorded between second and fourth parity (p<0.01).

As for the type of lambing, there was a significant difference between single and twin lambs (p<0.01), average weight for single lambs

recording a value of 4.33 ± 0.03 kg and for twin lambs 3.06 ± 0.05 kg (Table 2).
Sex of lambs was the non-genetic factor in which the differences between the two of groups was insignificant ($p>0.01$), females having an average BW of 4.02 ± 0.04 kg and male lambs 4.16 ± 0.04 kg (Table 2).

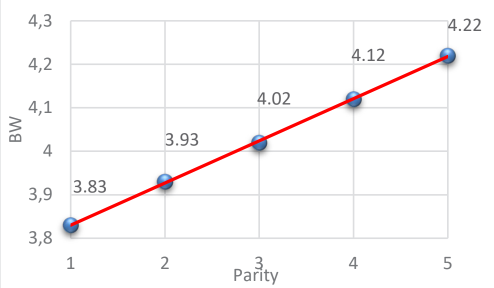

Figure 1. Scatter plot representing birth weight (BW) (kg) in relation to parity

Although male lambs were 0.14 kg heavier than females, statistically there were no significant differences ($p>0.01$) recorded that would have an effect on the variability of BW.
Analysis of variance results (Table 3) showed that all fixed effects, except sex of the lambs, significantly ($p<0.01$) contributed to the variability of the BW. The amount of variation in BW explained by the model was 42.99% ($R^2 = 0.4299$, $p<0.0001$).
This value is close to the values of other authors: Vlahek et al. (2021) who obtained a value of 33.79% for R^2; Gootwine and Rozov (2006) obtained an even higher R^2 (52%), however, they also included litter size, breeding group, season of birth, year of birth, gestation length as a covariable, month x parity interaction, and month x litter size interaction in the model (Vlahek et al., 2021).
In our model, this coefficient shows the percentage of the variation in the dependent variable that is explained by the independent variables included in the model (Starkings, 2012).

Table 2. Influence of non-genetic factors on the birth weight Teleorman Black Head lambs

Fixed factor	Level of factor	N	LSM ± SE (kg)
Sex	Female	407	4.02 ± 0.04
	Male	316	4.16 ± 0.04
Parity	1	243	3.83 ± 0.05
	2	97	3.93 ± 0.07
	3	91	4.02 ± 0.08
	4	222	4.12 ± 0.05
	5	70	4.22 ± 0.09
Lambing type	Single	591	4.33 ± 0.03
	Twin	132	3.06 ± 0.05

Results are presented as least square means and standard error (LSM ± SE)

Upon comparing our data to values reported in the literature, we have observed that male lambs have higher average birth weights than female lambs, and this trend is consistent among other sheep breeds, such as Romanov (Murphy et al., 2020; Vlahek et al., 2021); Assaf (Gootwine & Rozov, 2006), with 0.15, 0.19 and 0.22 kg higher average BW of males than females; Île-de-France (Achkakanova et al., 2020) with 0.07 kg; Dorper and Mutton Merino (Assan & Makuza, 2005) with 0.65 kg and 0.80 kg, respectively.
Additionally, the Blackhead Pleven Sheep breed, which is phenotypically very similar to the breed in our study, recorded, according to the authors Simeonov et al. (2014), a difference of 0.22 kg in average body weight at birth between males and females.
Differences in lamb birth weight depending on the parity of the ewes have been observed in breeds such as Assaf (Gootwine and Rozov,

2006), where the largest differences were between the first and fourth or fifth parity (0.53 kg); Romanov (Vlahek et al., 2021) between the first and sixth parity (0.55 kg); Macheri (Thiruvenkadan et al., 2008) between the first and fifth parity (0.25 kg); Doyogena sheep (Habtegiorgis et al., 2022) between first and fifth parity (0.23 kg). In our study, the biggest difference (0.39 kg) was identified between first and fifth parity (Table 4).

Table 3. Variance analysis of birth weight

Fixed factor	DF	MS	F	P	R (%)
Sex	1	3.34	5.39	0.02	
Parity	4	14.27	27.06	<0.001	42.99
Type of lambing	1	178.51	473.59	<0.001	

Regarding the differences in birth weights between single lambs and twin lambs, different values have been identified in many other sheep breeds, with the weight of single lambs being higher than those from multiple births, as expected.

Thus, values similar to the one obtained by us for the difference in average birth weight of lambs from the two types of births (1.27 kg) have also been identified in other breeds such as Dorper (0.02 kg), Mutton Merino (0.07 kg), Indigenous Sabi (0.23 kg) (Assan & Makuza, 2005); Romanov (0.55 kg) (Vlahek et al., 2021); Doyagena (0.76 kg) (Habtegiorgis et al., 2022); Blackhead Pleven (0.96 kg) (Simeonov et al., 2014); Assaf (1 kg) (Gootwine & Rozov, 2006).

Table 4. Analysis of birth weight differences based on parity

Parity interaction	**Mean**	**SE**	**P**
Parity 1-Parity 2	0.021	0.062	0.999
Parity 1-Parity 3	0.251	0.063	0.040
Parity 1-Parity 4	0.301	0.048	$9.1*10^{-5}$
Parity 1-Parity 5	0.315	0.070	0.012
Parity 2-Parity 3	0.272	0.075	0.078
Parity 2-Parity 4	0.321	0.062	0.003
Parity 2-Parity 5	0.336	0.080	0.027
Parity 3-Parity 4	0.050	0.064	0.982
Parity 3-Parity 5	0.064	0.082	0.981
Parity 4-Parity 5	0.014	0.070	1.000

Based on the obtained data, we can highlight that there is no significant effect of lamb gender on its birth body weight, even though male lambs usually have slightly higher birth weights than females. However, a higher birth weight leads to an increased degree of body development as they age, while it is also known that males have a higher average daily growth rate than females.

On the other hand, parity has a significant effect on birth weight. This may be due to the fact that higher parity indicates a greater age, which denotes a stronger body development and a better ability to use nutrients during gestation due to increased adaptability in this physiological state. This phenomenon can also be highlighted by the large differences between the parities of ewes in terms of birth weight of lambs (Table 4), a phenomenon observed in many other studies.

Lambing type is also a factor with a strong influence on birth weight of lambs. It is often influenced by the sheep breed. A larger number of lambs produced per lambing results in a higher litter weight but a lower weight per lamb.

A higher litter weight often leads to a low survival rate (Habtegiorgis et al., 2022), poor body development and a low average daily gain.

CONCLUSIONS

Birth weight is a trait influenced by both genetic and non-genetic factors. This study aimed to investigate the impact of non-genetic factors on birth weight in Teleorman Black Head sheep breed. The results showed that the average birth weight for the whole population of lambs was 4.11 ± 0.79 kg, and the, parity and type of birth significantly influenced birth weight ($p<0.01$) while sex of the lamb non-significantly ($p>0.01$).

The study highlights the importance of adjusting the non-genetic factors to obtain more accurate estimates of birth weight, which can be used to develop effective management strategies to enhance lamb productivity in this breed. Additionally, the study suggests that Teleorman Black Head sheep has good meat production performance due to its high birth weight and moderate-high daily gain.

In conclusion, birth weight is an important trait in the selection of breeding animals for the production of valuable offspring, providing information about the physiological status of the animal, its parents, and can also be used to estimate other traits of body development and production that are of biological and economic interest. This study is preliminary and was conducted on a relatively small sample; therefore, the development of studies related to this trait (BW) in breeds raised in Romania is necessary, especially for the Teleorman Black Head sheep breed, on which few research has been conducted and which proves to have high potential for expressing traits related to meat production.

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ASSESSMENT AND SELECTION OF COWS OF FUTURE MOTHERS OF SIMMENTAL BULLS WITH THE USE OF GENETIC MARKERS

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Abstract

There are given the results of the selection, the assessment of future mothers of bulls of the Simmental breed. The materials for the research were cows of the first, second and third lactation of the Simmental breed of the herd of the breeding farm Society of limited liability "Strapit". The studies were carried out according to the scheme developed by us according to the following parameters: origin and genetic examination; constitution and exterior, marker alleles; productivity; breeding value; genetic evaluation methods. Milk yields for 305 days of lactation on average for the population of Simmental cows amounted to 6017 kg of milk, which is by 455 kg of milk more than the average for the first lactation, the difference is significant (P<0.005). Alleles B_2I_1Q, I', I'Q' are markers for the Simmental breed. There were selected 4 cows as candidates for mothers of future bulls, whose productivity in terms of the highest lactation varied within 7029-8003 kg of milk.

***Key words**: assessment, blood groups, female ancestors, mothers of future bulls, productivity.*

INTRODUCTION

The breeding process requires intensive use of animals with outstanding performance, repeated in subsequent generations. The identification of such animals has become possible thanks to molecular genetic methods. Due to the rapid development of genetic technologies over the past two decades, the DNA technology method has become the main method for improving the use of farm animals (Glazko et al., 2017; Kgwatalala et al., 2007).

Improvement in the methodology of biology and molecular genetics made it possible in 2010 to decipher the genome of the most important species of agricultural animals - cattle, pigs, sheep and to genotype the studied species using hundreds of DNA markers. Of the majority of genetic markers, the most informative and most convenient for practical use was SNP (Single Nucleotide Polymorphism), (Kgwatalala et al., 2007; Matukumalli et al., 2009; VanRaden et al., 2010; Tracovicka, 2015).

The development and implementation of genomic evaluation marked the beginning of a new era in dairy cattle breeding. Selection based on genomic evaluation does not take place on individual "master genes", but on the entire genome. Knowing the DNA sequence of the father and mother makes it possible to predict which parts of the genome and hereditary inclinations a descendant can receive. According to the data of the Holstein and Jersey associations, the addition of genomic information increases the reliability of the assessment of bulls for all indicators, and the accumulation of data leads to an increase in the accuracy of the "genomic prediction" (Kalashnikova, 2010).

However, the successes of molecular genetics "pushed" into the background such a section of genetic research as immunogenetic. According to Serdyuk (2018), referring to Altukhov's research, in his article notes that a similar state of affairs is erroneous, since..."Polymorphism of proteins, blood groups and DNA naturally complement each other..." (Legarova, 2010). Therefore, the use of allelic forms of genes responsible for blood groups is also very relevant.

In the Republic of Moldova, as a result of many years of research in the breeding of the Moldovan type of black-motley cattle, information has been accumulated on the blood groups of cattle of various breeds (Smirnov et al., 2007). It was created a database of the allele

pool of the EAB-locus of the Moldovan type of black-motley cattle on the basis of attestation materials for blood groups of bulls belonging to 18 lines of the Moldovan type of black-motley cattle (Foksha et al., 2008).
Alleles of the AEB locus were used in the choice and selection of bulls and breeding stock, where genetic markers of both bulls and breeding stock are taken into account (Konstandoglo et al., 2015).
Taking into account the difficulties in the development of breeding cattle in the Republic of Moldova, it is important to obtain breeding bulls and heifers to further increase the genetic progress in dairy cattle breeding.
The aim of the research was to evaluate cows of the Simmental breed (future mothers of bulls) by origin and their own productivity using blood groups, to obtain bulls from them in the future for growing on the Elever.

MATERIALS AND METHODS

The research was carried out in the herd of Society of Limited Liability "Strapit" (LLC "Strapit"), where Simmental animals are bred, which were imported from Austria and Germany.
The material for the research were the cows of the first, second and third lactation of the Simmental breed of the herd of the breeding farm LLC "Strapit", v. of Nitscani, district of Calarash.
Selection, testing and immunogenetic marking of mother cows- bull producers from the Simmental breed were carried out using our own elaborations (Smirnov & Konstandoglo, 2009) (Figure 1).
The research carried out was directed at the testing of Simmental breed cows (future mothers producing bulls) on genealogical indices, own production, immunogenetic testing of blood groups (n = 64). An extensive analysis of the records of the population of Simmental bulls, the register of the growth and development of young bulls, the registers of cattle appraisal, the control milking and selected according to the scheme previously developed by the laboratory collaborators, were carried out.
The collection of blood samples, the performance of testing and the study of blood groups was carried out according to the recommendations of genetic testing (1983). The frequency of antigens and alleles was calculated according to the recommendations of Mierkuriev (1983). The level of homozygosity (Cα) was calculated using the Robertson formula: $Ca = \Sigma p^2$, where Ca - the homozygosity level at one locus, p - the allele frequency at the locus.
For the studied traits, the arithmetic mean (M), the error of the mean (± m), the coefficient of variation (Cv), and the significance of the difference according to Student's criterion (P) were determined. Statistical data processing and correlation analysis were carried out according to Merkurieva & Shangin-Berezovsky (1983) using the Microsoft Excel 2010 software package.

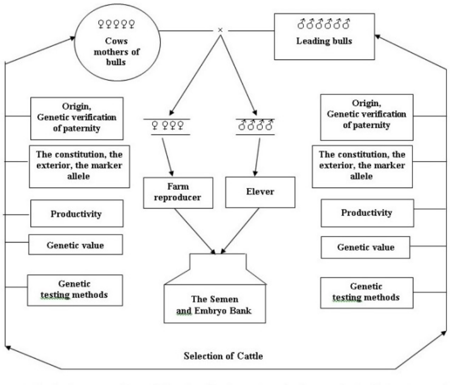

Figure 1. The scheme of selecting the cows-mothers of bulls and performing nominated pairings to obtain offspring for growth and reproduction

RESULTS AND DISCUSSIONS

The selection and evaluation of future mothers of Simmental bulls in the herd of LLC "Strapit" was carried out according to the following parameters: lineage and genetic expertise; constitution and exterior, marker alleles; productivity; breeding value; genetic evaluation methods.
Assessment and analysis of the productivity of the female ancestors of the Simmental heifers of the German selection showed that the milk yield of cow mothers averaged 7660 kg of milk with a fat content of 4.23% (Table 1).

Table 1. Productivity of female ancestors of the Simmental breed (German selection) (X ± Sx)

Indicators	Mother	C_v, %	Father's mother	C_v, %	Mother's mother	C_v, %
Milk yield, kg	7660 ± 198.1	17.3	11007 ± 239.1	15.2	8188 ± 202.0	16.9
Fat, %	4.23 ± 0.05	8.2	4.18 ± 0.07	11.3	4.10 ± 0.05	9.0
Fat, kg	323 ± 8.6	17.9	459 ± 11.4	17.4	336 ± 9.0	18.4

The milk yield of mothers and fathers according to the highest lactation amounted to 11007 kg of milk with a fat content of 4.18%.
The coefficients of variability (Cv) for milk yield were slightly above the lower limit of the norm for all analyzed groups of mothers from 15.2% (mothers of fathers) to 17.3% (mothers). In terms of fat content, the coefficient of variability was within the normal range from 8.2% (mothers) to 11.3% (mothers of fathers). As for the amount of milk fat, it should be noted that the values of this trait at mothers and mothers of fathers were below the norm, and at mothers of mothers they corresponded to the literature data (18.4%). Thus, the obtained results of the coefficients of variability and their comparison with the literature data led to the conclusion about a wide range of their variability and high genetic diversity of the analyzed population of Simmental cattle.
An analysis of the pedigrees of breeding evidence showed that heifers of the Simmental breed of German selection were inseminated by different bulls. The largest number of offspring (4) was found at the bull of the Austrian selection Pandora AT 597742517, at the bull of the German selection Dryland DE 09 4553211 (2), the productivity of mothers of which for the highest lactation was 12117 and 13049 kg of milk, with a fat percentage of 4.59 and 4.75 respectively. The mother of the bull Ilja DE 09 42492282 has the highest fat content in milk - 5.36%, with a milk yield for the highest lactation of 8739 kg of milk, the number of daughters is - 2.
The breeding value of bulls in terms of milk productivity on average for the analyzed livestock was +640.7 kg of milk, that is, all bulls are improvers in terms of milk yield.
As for the presence in the pedigrees of bull's genotypes from different countries, it was established as a result of the assessment that the origin of the fathers - producers were from two countries: Germany (82%) and Austria (18%) (Figure 2).

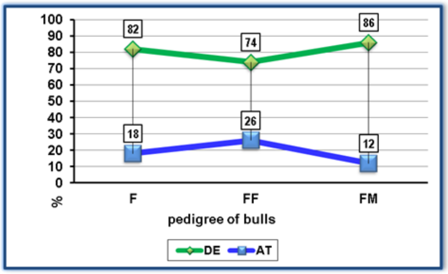

Figure 2. Genotypes from different countries as part of the parents of producers
Note: F - father; FF - the father of the father; FM - the father of the mother

In the FF and FM genotypes, there are 74 and 86% of bulls from Germany, 26 and 12%, respectively, from Austria.
The next stage of the research was to evaluate the analyzed cows of the Simmental breed according to their own productivity (Table 2).

Table 2. Characteristics of Simmental cows in terms of milk production for 305 days of lactation, (X ± Sx)

Lactation	Number of cows, n	Milk yield, kg	Cv	Fat content, %	Cv	Amount of Fat, kg	Cv
The first	10	5562 ± 179.5	9.7	3.94 ± 0.02	1.3	219 ± 7.1	9.8
The second	I23	6191 ± 106.2***	8.2	3.95 ± 0.01	1.4	249 ± 6.6	12.7
The third	19	5990 ± 212.5	15.5	3.98 ± 0.01	1.3	238 ± 8.0	14.7
Average population	52	6017 ± 102.3*	12.4	3.96 ± 0.01	1.4	240 ± 4.5	13.8

It was found that milk yield for 305 days of lactation on average for the population of Simmental cows was 6017 kg of milk, which is by 455 kg of milk more than the average for the first lactation, the difference is significant ($P<0.005$). It should be noted that the comparison of milk yields between the second and first lactations showed an increase of 629 kg of milk ($P<0.001$). Depending on lactation, the lowest coefficient of variability in milk yield per lactation was at cows of the second lactation - it amounted 8.2%, which is by 7.3 and 4.2% less than at cows of the third lactation and on average for sample, respectively. According to the content and amount of milk fat in milk, the coefficient of variability is lower compared to the literature data.

The next stage of research is genetic marking by blood groups. It is known that the genetic information about animals, represented by blood groups, is used repeatedly, including after the disposal of the animal. Next, we present a detailed description of the antigenic spectrum, the allelophond of the AEB locus of certified Simmental animals according to blood groups.

As a result of the research, it was found that the antigenic spectrum of blood groups of the analysed animals of the Simmental breed turned out to be quite wide and is characterized by a greater saturation of antigenic factors due to the high frequency of occurrence of antigens V, H′, Z (Table 3).

Table 3. Antigenic spectrum of blood groups of Simmental cows, LLC "Strapit"

No	Locuses	Antigens	n	Frequency of antigens	No	Locuces	Antigens	n	Frequency of antigens
1.	**A**	A_2	45	0.6923	25.	**C**	C_1	34	0.5231
2.		Z'	1	0.0154	26.		C_2	34	0.5231
3.	**B**	B_2	35	0.5385	27.		E	45	0.6923
4.		G_2	30	0.4615	28.		R_1	4	0.0615
5.		G_3	32	0.4923	29.		R_2	30	0.4615
6.		I_1	24	0.3692	30.		W	63	0.9692
7.		I_2	19	0.2923	31.		X_1	9	0.1385
8.		O_2	18	0.2769	32.		X_2	38	0.5846
9.		P_2	3	0.0432	33.		C′	10	0.1538
10.		Q	20	0.3077	34.		L′	25	0.3846
11.		T_1	8	0.1231	35.	**F-V**	F	65	1.0000
12.		T_2	7	0.1077	36.		V	40	0.6154
13.		Y_2	22	0.3563	37.	**J**	J_2	30	0.4615
14.		D'	12	0.1846	38.	**L**	L	26	0.4000
15.		E'_2	23	0.3538	39.	**M**	M	8	0.1231
16.		G'	27	0.4154	40.	**S**	S_1	47	0.7231
17.		I'	24	0.3692	41.		U	2	0.0308
18.		J'_2	2	0.0308	42.		H′	63	0.9692
19.		O'	21	0.3231	43.		U′	9	0.1385
20.		P'	15	0.2308	44.		H″	7	0.1077
21.		Q′	36	0.5538	45.		U″	23	0.3538
22.		Y′	4	0.0615	46.	**Z**	Z	58	0.8923
23.		B″	3	0.0462	Average frequency or saturation of antigenic factors				0.2974 29.7%
24.		G″	21	0.3231					

The high frequency of occurrence of antigens A_2, E, W, V, S_1, H′, and Z should be noted. Similar results were obtained in studies of Austrian breeding Simmental cows (Sakhautdinov et al., 2011), where a high frequency of occurrence of the above antigens was also revealed.

For the AEA locus, both antigens were identified, the frequency of occurrence of which is 0.6923 (antigen A_2) and 0.0154 (antigen Z′).

All antigens were identified for the AEB locus out of 22 studied. In the tested population of animals, the most common antigens were B_2, G_2, G_3, I_1, Y_2, G', Q' (Figure 3).

Our data confirm studies (Gumerov, 2009; Didyk, 1987), the authors identified similar antigens of the AEC locus with a high frequency of occurrence inherent to the Simmental breed.

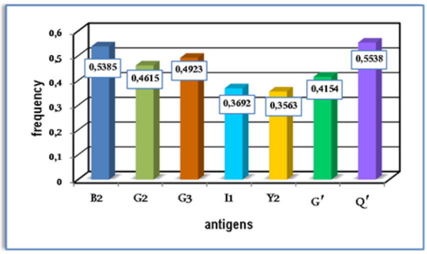

Figure 3. Frequency of occurrence of AEB locus antigens

The obtained results are consistent with the results of studies in the population of Simmental cattle in the herd of Technologically Experimental Station "Maksimovka" (Konstandoglo & Foksha, 2008), as well as the previously obtainned results of testing the Simmental population of the analyzed herd (Konstandoglo et al., 2016). The frequency of occurrence of antigens I_2, O_2, Q, Y_2, D', O', P' varied from 0.1846 (D' antigen) to 0.3563 (Y_2 antigen). Further, the antigens T_1 and T_2 were distributed in descending order with a frequency of occurrence of 0.1231 and 0.1077, respectively. The most common among animals of the Simmental breed on the AEC locus turned out to be antigens C_2, E, W, X_2, which are characteristic of this breed.

According to the F-V- locus, both antigens were detected in the entire analyzed population of animals. The frequency of occurrence of antigen F was 1.0, antigen V - 0.6154.

In one-factor AEJ-, AEL-, AEM-, AEZ-loci, all studied antigens were revealed. The frequency of occurrence of antigens J_2 and Z is high and is 0.4615 and 0.8923, respectively. Carriers of antigen M among the tested animals are more than 12%, the frequency of which is 0.1231.

All antigens were identified for the AES locus, the most common were antigens S_1, H', U″. The frequency of occurrence of the H' antigen is quite high and amounts to 0.9692.

An assessment of the saturation of the analyzed animals with antigenic factors showed that at the animal population of the Simmental breed it is 29.7%. As it is known, the alleles of the AEB locus to a greater extent reflect the hereditary characteristics of animals and characterize their genetic diversity.

As a result of the studies, 66 alleles were identified of AEB-locus of the Simmental breed (Table 4).

Table 4. Genetic structure of the Simmental breed of AEB locus

No	Alleles	Number of alleles	Frequency	No	Alleles	Number of alleles	Frequency
1.	B_2G_2	11	0.0846	34.	$Y_2D'E'_2Y'$	1	0.0080
2.	$B_2G_2I_1Q$	2	0.0154	35.	$Y_2G'IQ'$	1	0.0080
3.	$B_2G_2I_1O_2QT_1$	1	0.0080	36.	$Y_2G'IQ'G'$	1	0.0080
4.	$B_2G_2O_2$	1	0.0080	37.	$Y_2E'_2G'I'O'P'G''$	1	0.0080
5.	$B_2G_2O_2T_1$	1	0.0080	38.	$Y_2E'_2G'I'O'Q'G''$	1	0.0080
6.	$B_2G_2O_2P_2Q$	1	0.0080	39.	$Y_2E'_2I'O'Q'$	1	0.0080
7.	$B_2G_2P_2Q$	1	0.0080	40.	Y_2G'	1	0.0080
8.	$B_2G_2T_1$	1	0.0080	41.	$Y_2G'I'$	1	0.0080
9.	$B_2G_2I'OT_1$	1	0.0080	42.	Y_2I'	2	0.0154
10.	$B_2G_3I_1$	1	0.0080	43.	$Y_2I'P'Q'$	2	0.0154
11.	$B_2G_3T_1$	2	0.0154	44.	$Y_2I'P'Y'$	1	0.0800
12.	B_2I_1	1	0.0080	45.	$Y_2I'Q'$	4	0.0308
13.	$B_2I_1P_2Q$	1	0.0080	46.	$D'E'_2G'O'G''$	1	0.0080
14.	$B_2I_1O_2Q$	1	0.0080	47.	$D'E'_2G'O'P'G''$	1	0.0080
15.	B_2I_1Q	6	0.0461	48.	$D'E'_2I'J'_2O'$	1	0.0080
16.	B_2O_1Q	1	0.0080	49.	$D'G'I'Q'G''$	1	0.0080
17.	$B_2O_2Y_2E'_3G'Q'G''$	1	0.0080	50.	$D'G'J'_2P'$	1	0.0080
18.	B_2QG'	1	0.0080	51.	$E_2'G'I'O'$	1	0.0080
19.	G_2	2	0.0154	52.	$E_2'G'I'O'G''$	3	0.0230
20.	G_2I_1	1	0.0080	53.	$E_2'G'I'O'P'G''$	2	0.0154
21.	$G_2O_2T_1$	2	0.0154	54.	$E_2'G'I'O'Q'G''$	2	0.0154
22.	G_2T_2	2	0.0154	55.	$E_2'G'O'P'G''$	1	0.0080
23.	G_2Y_2	1	0.0080	56.	$E_2'G'O'P'Q'G''$	1	0.0080
24.	I_2	3	0.0230	57.	$E_2'G'O'G''$	1	0.0080
25.	I_1Q	1	0.0080	58.	$E_2'I'$	1	0.0080
26.	O_1I'	1	0.0080	59.	$E_2'I'O'Q'$	1	0.0080
27.	O_2	9	0.0692	60.	$G'I'$	2	0.0154
28.	Q	3	0.0231	61.	$G'O'G''$	1	0.0080
29.	Y_2D'	1	0.0080	62.	I'	7	0.0538
30.	$Y_2D'G'I'Q'Y'B''$	1	0.0080	63.	$I'Q'$	9	0.0692
31.	$Y_2D'G'Q'Y'B''$	1	0.0080	64.	Q'	9	0.0692
32.	$Y_2D'E'_2I'O'Q'$	1	0.0080	65.	G''	1	0.0080
33.	$Y_2D'E'_2I'P'$	1	0.0080	66.	"b"	1	0.0080

The most common alleles were B_2G_2, B_2I_1Q, O_2, I', $I'Q'$, Q'. It should be noted that the alleles B_2I_1Q, I', $I'Q'$ are markers for the Simmental breed.

Alleles B_1I_1Q, I_1Q, O_1I', Q, $I'Q'$, Q' "b" are common for breeds of "pale-motley" root - Sychevskaya, Charolais, Pinzgau.

The objective genetic characteristics of the analyzed animals of the Simmental breed are also reflected by such indicators as the homozygosity coefficient (Ca), the number of effective alleles (Na) (Table 5).

Table 5. Genetic variability of the Simmental cattle population

No	Indicators	Quantitative value
1.	Total explored, heads	65
2.	Number of established alleles: - total - main - rare	128 84 44
3.	Total allele frequency: - basic - rare	0.6461 0.3385
4.	Homozygosity coefficient, Ca	0.0351
5.	Number of effective alleles, Na	28.5

As it can be seen, the concentration of the main alleles for the Simmental breed in the herd of LLC “Strapit” was 64.6%. The homozygosity coefficient in the analyzed population is low and amounts to 3.5%, which indicates a high genetic diversity of this population of Simmental cattle. As it is known, the state of the allelophond of the breed in terms of the level of homozygosity is reflected in the indicator of the number of effective alleles. Research have established that in the population of animals of the Simmental breed of the herd of LLC “Strapit”, the number of effective alleles reaches 28, which corresponds to the maximum possible "homozygous" structures in the herd and reflects the state of heterozygosity for this locus.

In general, the allelophond of the examined animal population of the Simmental breed is dominated by marker allele’s characteristic of the Simmental cattle of Austrian and German breeding, and the identified differences.

Thus, as a result of assessing the of Simmental cows by origin (productivity of female ancestors, breeding value of bulls) and their own productivity, as well as certification by blood groups (detailed characteristics of the antigenic spectrum and allelophond of the AEB locus), 4 cows were selected, which we offer candidates for future bull-producing cows (Table 6).

Table 6. Characteristics of selected cows as mothers of future bulls

No	Number	Lactation	Milk yield, kg	Fat content, %	Amount of fat, kg	Alleles of the AEB locus
1.	MD 76405	The third	8003	3.94	315.3	$B_2O_2Y_2E'_3G'Q'G''/E'_2G'I'O'$
2.	MD 76399	The second	7029	3.86	271.3	$G_2Y_2/Y_2I'P'Y'$
3.	MD 8125	The third	7285	3.93	286.3	$Y_2G'I'Q'/G''$
4.	MD 22729	The second	7465	3.9	291	$B_2G_2/E'_2G'O'G''$

As it can be seen, all the candidates for mothers of future bulls are the same age, the productivity of which, according to the completed highest lactation, varied within 7029-8003 kg of milk. The selected cows are marked with alleles of the AEB locus and are valuable in obtaining bulls with a high genetic potential, well adapted to the breeding conditions of the Republic of Moldova.

CONCLUSIONS

1. Milk yield for 305 days of lactation on average for the analyzed population of Simmental cows amounted to 6017 kg of milk, which is by 455 kg of milk more than the average for the first lactation, the difference is significant ($P<0.005$).
2. Alleles B_2I_1Q, I', $I'Q'$ are marker alleles for the Simmental breed.
3. Candidate cows in mothers - producers of bulls have the average milk production during the highest lactation in the range of 7029-8003 kg milk per lactation, are genetically marked and show value for obtaining autochthonous bulls of high genetic value well adapted to the pedoclimatic conditions of Republic of Moldova.

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ESTIMATION OF CORRELATION COEFFICIENTS BETWEEN MILK YIELD AND MORPHOLOGICAL TRAITS IN A POPULATION OF LACAUNE SHEEP

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Abstract

The aim of the study was to estimate correlations between milk yield and growth traits. The biological material was 282 Lacaune ewes and traits studied were: milk yield (MY), body weight (BW), height at withers (HW), height at rump (HR), torso length (TL), chest width (CW), rump width (RW), chest depth (CD), chest girth (CG) and metacarpal circumference (MC). The data obtained were statistically processed and interpreted. Average values for milk yield was 120.86 kg for 135 days; average values for morphological traits was 65.10 kg for BW, 62.48 cm for WH, 63.23 cm for RW, 55.25 cm for TL, 25.04 cm for CW, 23.60 cm for RW, 24.06 cm for CD, 84.07 cm for CG and 6.91 cm for MC. Correlations were determined by Pearson test and correlation coefficients had values of +0.13 between MY x BW, -0.02 between MY x WH, -0.19 MY x RH, +0.12 between MY x TL, -0.03 between MY x CW, -0.08 between MY x RW, +0.03 between MY x CD, +0.002 between MY x CG and -0.06 between MY x MC.

***Key words**: body traits, correlation coefficient, Lacaune, milk yield.*

INTRODUCTION

At the moment, there is an upward trend among consumers to purchase high-quality food products, ecologically produced and from local producers. Sheep milk and dairy products can represent some of these foods that show a growing demand from the population of European countries and beyond.

In Romania, despite a tradition of sheep breeding, supported by the large number of sheep raised nationally - 10.1 million in 2021 (FAO, 2021), the consumption of products obtained from this species is relatively low but with some growth in recent years. In this context, it is considered necessary to study sheep populations to identify methods for conserving specialized breeds for milk production and improving their production.

The success of milk production selection in sheep, as well as in other species, lies not only in the direct improvement of this complex of traits (milk quantity, fat or protein content in milk), but also in the improvement of other traits related to body development or functional characteristics of the organism (Žujović et al., 2011). On the other hand, the determination of absolute body dimensions is also very important, as these represent the morphological and physiological basis not only for highlighting body development but also for optimal expression of production and reproductive traits (Riva et al., 2004, cited by Žujović et al., 2011). Considering these aspects, the study of correlations between milk production and body development becomes important. Determining body dimensions can provide essential information on the course of animal development as well as the expression of certain traits.

The purpose of this article is to highlight the correlations between quantitative milk production and certain body dimensions in order to use the data in selection methods for valuable breeding animals. At the same time, determining body dimensions in Lacaune sheep is rarely emphasized in the specialized literature.

The choice of studying this breed of sheep derives from the fact that the population is small compared to those of native breeds raised in the country (Tsurcana and Tsigai), but the milk production is significantly higher compared to these breeds.

MATERIALS AND METHODS

The biological material consisted of 282 Lacaune ewes included in the Official Performance and Recording Scheme (COP), on which the total milk yield obtained from the tests was calculated using the technical rules of the International Committee for Animal Recording (ICAR). In our case, we used the alternating monthly test (AT) method, which involves measuring the amount of milk recorded for each sheep in the flock during one of the two daily milkings, and the average recording interval is monthly (30 days) (ICAR Guidelines, 2018). The total control period for the studied flock was 120 days, which includes four milk tests, carried out after the weaning period. The amount of milk calculated at the end of the tests was expressed in liters and represented by total milked milk (TMM), which represents the milk yield produced during the milking-only period (ICAR Guidelines, 2018). The milking was done by machine, and the quantitative recording for each ewe was determined at each test.

In the studied farm, during the winter period, ewes are fed exclusively with quality hay, mash, grains, and concentrates, which are administered on the feed alleys located in the middle of the shelter. During the summer, the forage is of mixed type, so in the warm and rainless periods, the animals are grazed on the plots intended for this purpose, and in the cold and rainy periods, the sheep are fed with hay and mowed green grass along with the concentrates supplement.

Regarding body measurements, they were determined at the third milk test in the morning, before introducing the sheep for milking. The tools used for measurement were an electronic scale, measuring stick, compass, and tape measure. The morphometric traits determined were as follows: body weight (BW); height at withers (HW) - vertical distance from the highest point of the interscapular region to the soil (Riva et al., 2004); height at rump (HR) - the vertical distance from the top of the pelvic girdle to the ground (Cam et al., 2010); torso length (TL) - length from the anterior shoulder point to the posterior extremity of the pin bone (Cam et al., 2010); chest width (CW) - distance between scapulae tubers; rump width (RW) - the distance between coxal tubers; chest depth (CD) - distance from the highest point of the interscapular region to the lowest point of sternum; chest girth (CG) (or heart girth as stated by Cam et al., 2010; Carneiro et al., 2010; Stanjko et al., 2010; Riva et al., 2004) - largest circumference of the thorax, passing by the sternum, and metacarpal circumference (MC) (Azarpajouh et al., 2021) or cannon bone circumference (Anila et al., 2021; Sun et al., 2020).

Body weight was determined using an electronic scale; height, length, and some width dimensions were determined using a measuring stick (Lydthin's rod) (Stajnko et al., 2010). A compass was also used for width measurements, and a metric tape was used for circumference measurements.

The data analysis for this paper was generated using the Real Statistics Resource Pack software (Release 7.6), Copyright (2013-2022) Charles Zaiontz. The variability of the analyzed traits was described using descriptive statistical parameters, and correlations between traits were determined using the Pearson correlation coefficient.

RESULTS AND DISCUSSIONS

Basic parameters of descriptive statistics for milk yield and individual measures of body development (Table 1) showed that the small values of SD and CV% obtained for the majority of parameters indicates that the studied population is a uniform one in terms of milk yield and morphological variability. The average milk yield during the 120-day lactation and control period was 120.86 ± 8.59 kg, values close to those obtained by Libis-Márta et al., who recorded an average milk production of 197.72 ± 37.43 kg over a 229-day lactation period. Following the measurements taken to determine BW, HW, HR, TL, CW, RW, CD, CG, and MC, mean values of 65.1 ± 3.76 kg, 62.48 ± 2.75 cm, 63.23 ± 3.17 cm, 55.25 ± 3.20 cm, 25.04 ± 2.35 cm, 23.60 ± 2.35 cm, 24.06 ± 2.14 cm, 84.07 ± 3.52 cm and 6.91 ± 0.52 cm, respectively, were obtained. These values highlight the belonging of the studied sheep population to the dairy type, a fact suggested in other studies on other dairy sheep breeds, such as Frizarta (Kominakis et al., 2009) and Spanish Asaaf (Ángeles Pérez-Cabal et al., 2013), where near values were determined.

Table 1. Average values and variability of milk performance and morphometric traits of Lacaune sheep breed

Traits	N	$\bar{x}$	SD	CV %	Min.	Max.
MY (kg)	282	120.86	8.59	7.11	98.68	144
BW (kg)	282	65.1	3.76	5.78	57.16	72.88
HW (cm)	282	62.48	2.75	4.41	57.06	68.96
HR (cm)	282	63.23	3.17	5.02	57.20	70.98
TL (cm)	282	55.25	3.20	5.79	49.03	61.97
CW (cm)	282	25.04	2.35	9.40	20.15	29.91
RW (cm)	282	23.60	2.35	9.96	19.01	28.75
CD (cm)	282	24.06	2.14	8.88	20.11	27.96
CG (cm)	282	84.07	3.52	4.19	77.17	90.64
MC (cm)	282	6.91	0.52	7.49	6.00	8.00

$\bar{x}$ - average, SD - standard deviation, CV% - coefficient of variation, MY - milk yield, BW - body weight, HW - height at withers, HR - height at rump, TL - torso length, CW - chest width, RW - rump width, CD - chest depth, CG - chest girth, MC - metacarpal circumference

The correlation is one of the most common and useful statistics that describes the degree of relationship between two variables. (Sun et al., 2020).
Regarding the obtained results, positive values of milk production correlations were identified in relation to BW (r = 0.13, p<0.05), TL (r = 0.12, p<0.05), CD (r = 0.038, p>0.05), and CG (r = 0.002, p>0.05), where the p value showed that the level of correlations was significant for BW and TL, but not significant for CD and CG. Negative correlations were not significant between MY and HW (r = -0.02, p>0.05), MY and CW (r = -0.03, p>0.05), MY and RW (r = -0.086, p>0.05), but strongly significant between MY and HR (r = -0.19, p<0.01) (Table 2).

Table 2. Results relating to study of the strenght of correlation between milk performance and certain body development measures of Lacaune sheep breed

r	MY (kg)	BW (kg)	HW (cm)	HR (cm)	TL (cm)	CW (cm)	RW (cm)	CD (cm)	CG (cm)	MC (cm)
MY										
BW	0.13*									
HW	-0.02ns	0.21***								
HR	-0.19**	0.09 ns	0.17**							
TL	0.12*	0.19**	0.16**	0.200***						
CW	-0.03ns	0.05 ns	0.24***	0.248***	0.21***					
RW	-0.09ns	0.15*	0.13*	0.251***	0.11ns	-0.008ns				
CD	0.04ns	-0.004 ns	-0.05ns	0.074ns	-0.01ns	-0.008ns	0.13*			
CG	0.002ns	0.004 ns	0.04ns	0.025ns	-0.04ns	0.06ns	0.05ns	-0.05ns		
MC	-0.06ns	0.05ns	-0.02ns	-0.095ns	-0.11ns	-0.06ns	-0.01ns	0.03ns	0.03ns	

* = p<0.05, ** = p<0.01, *** = p<0.001 NS = non significant; MY - milk yield, BW - body weight, HW - height at withers, HR - height at rump, TL - torso length, CW - chest width, RW - rump width, CD - chest depth, CG - chest girth, MC - metacarpal circumference

The simple regression equations regarding the relationship between MY and the other nine morphometric traits of Lacaune sheep breed population studied (Figure 1) returned values which show that body weight is the most highly related to milk yield.

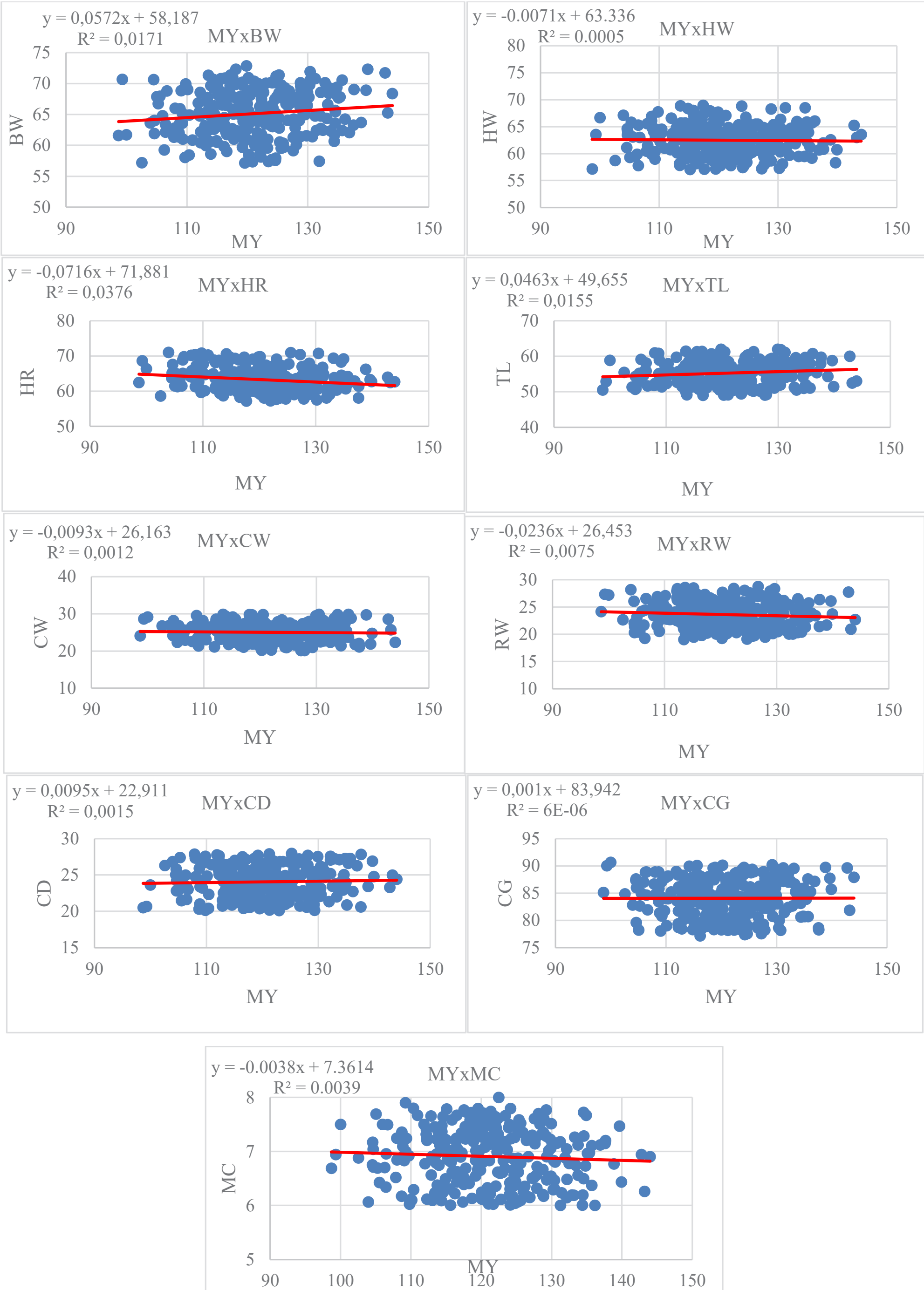

Figure 1. Relationship between milk yield and the studied morphometric traits of Lacaune sheep breed population

Regarding the positive correlation between milk production and body weight, Angeles Hernandez et al. obtained close values (r = 0.44) in the East Friesian sheep breed. Additionally, Gootwine (2011), in his genetic improvement program for the Awassi breed, reported that higher milk production is associated with an increase in sheep body mass.

Other positive values of correlations between MY x BW (r = 0.48, r = 0.132), (r = 0.48) were also determined in some goat breeds in the works of Iloeje and Van Vleck (1978) and respectively Žujović et al. (2011).

However, some studies (Mavrogenis and Papachristoforou, 2000 cited by Angeles Hernandez et al., 2018) suggest that body weight is not a determinant of milk volume produced in Chios sheep and Damascus goats, as they obtained very low genetic and phenotypic correlations (r = 0.08). Some authors (Berry et al., 2007) suggest that these low values may be a feed management-related characteristic.

The correlations between all body measurements varied in the range of -0.11 to 0.25. The strongest positive correlations were identified between HR x CW, HR x RW, and BW x HW.

Other authors (Sun et al., 2020; Anila et al., 2021) obtained similar correlation values between the same traits, but they obtained higher values between other traits (i.e. BW x HW: r = 0.62, r = 0.87) in Jamuna sheep breed and Bardhoka sheep breed, respectively. The strongest negative correlations were found between TL x MC and TL x HR.

The low values of some correlation coefficients between morphometric traits compared to literature values may be due to the differences between age and parity of the studied ewes which could have influenced some of the measurements , the small number of animals or calculation errors.

CONCLUSIONS

The results shows that certain morphometric traits: body weight, trunk length, chest depth, and trunk circumference have a direct effect on the milk production of the sheep population studied. Dimensions such as wither height, trunk width, rump width, and pin bone circumference have negative but insignificant correlations with milk production. In this case, we can conclude that in the selection of individuals for reproduction, body weight and trunk length can be taken into account as heritable traits that could have an effect on the future milk production of the offspring from the studied sheep population.

The regression analysis showed that milk yield could not be predicted from body morphometric trait data alone. However, when correlated with other information such as pedigree and environment, these traits can become an important tool for breeders and farmers to select the best individuals for breeding and increase milk yield.

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PRODUCTIVE QUALITIES OF COWS OF THE HOLSTEIN BREED OF DIFFERENT ORIGIN

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Abstract

There are presented the results of the studying productive qualities of Holstein cows of various origins. The material for the research was cattle of the Holstein breed of German selection- Society of limited liability "Dastocom" and French selection - Society of limited liability "Holstein". Milk yield of cows in the herd of LLC "Dastocom" for the first lactation averaged 8851 kg of milk. The milk productivity of Holstein cows of French selection (LLC "Holstein") for the first lactation averaged 6334 ± 34.7 kg of milk. At cattle of the Holstein breed of German selection (LLC "Dastocom"), a high level of heritability for milk yield (mother-daughter) was found, which amounted to 0.756. The greatest influence on the fat content in the milk of the first-calving cows of LLC "Dastocom" was exerted by paternal ancestors (h^2 = 0.39). For the entire analyzed population of cows-heifers of LLC "Holstein", milk yield and fat content in milk were largely determined by heredity (h^2 = 0.24-0.49) and (h^2 = 0.44-0.32), respectively.

***Key words**: correlation, fat content, heritability, milk yield, variability.*

INTRODUCTION

In dairy cattle breeding of developed countries of the world, the highly productive Holstein breed occupies a leading position. The use of highly productive animals in breeding work contributes to the accumulation of the most valuable genetic potential of cows, increases the possibility of obtaining even more highly productive breeding herds (Nemtseva, 2019; Stepanov et al., 2019; Zernina, 2019; Zyryanova, 2018).

Of great importance for the theory and practice of breeding is the use of heritability coefficients (Kibkalo et al., 2004). Currently, there are enough reports in the zootechnical literature about the limits of variation in the coefficient of heritability of milk productivity traits (Kuznetsov et al., 2002; Lepekhina, 2012; Nazarchenko, 2012).

In the course of the work of these researchers, a very important conclusion follows that if a single lactation, in relation to which it is known that it has a relatively small heritability coefficient, it says little about the breeding value of a cow, then the information about her milk yields, generalized for five lactations, already give a fairly complete representation for judging its breeding value. It was also noted that with an increase in the level of herd productivity, the heritability coefficient also increases, and as the age of cow's increases, heritability tends to decrease (Mistzal et al., 1993).

Evaluation of the influence of mother cows on the yield and quality composition of offspring milk is one of the leading prerequisites for breeding cattle (Damarov et al., 2018; Kozlov, 2019; Kulikova et al., 2016; Piotrovskaya et al., 2018; Titova, 2018;).

For the last decades, Holstein cattle have been imported to the Republic of Moldova from such European countries as Holland, Germany, France, Austria and Hungary. These animals are distinguished by increased productivity, good health and are able to acclimatize and adapt to the conditions of various climatic zones of the republic. Holstein cattle purchased from Holland and Germany have successfully adapted to the conditions of the south of the Republic of Moldova, in particular in the herds of Joint-Stock Company "Aydyn" and Society of Limited Liability "Doksancom". It should be noted that under the conditions of free-range maintenance and balanced feeding, the milk of Holstein cows of Dutch breed in the herd of Society of Limited Liability “Doksancom” averages 9085 kg of milk with a fat content of 3.88% after the first lactation (Foksha et al., 2021). Milk yields of breeding cows of the Holstein breed in the herd of Joint-Stock Company “Aydyn” produced 10,560 kg of milk with a fat content of 3.85% (Dutch breed) and 10,102 kg of milk with a fat content of 3.92% (German breed) in the third lactation (Foksha et al., 2020).

The aim of the research is to study the genetic and population processes at cows of German and French breed and to reveal the correlations and heritability of the main economically useful traits.

MATERIALS AND METHODS

The research material was Holstein cattle of German breed – Society of Limited Liability “Dastocom”, v. Styrcha, Glodyany district and French breed – Society of Limited Liability “Holstein”, v. Roshkan, Aneniy Noy district. The milk yield for 305 days of lactation, the fat content in milk and the amount of milk fat were studied, the variability of these indicators (Cv) was determined.

The genetic potential of the productivity of primiparous cows was determined on the basis of the parental index of cows (PIC). Parental indices were calculated by the Wright path coefficient (Krasota & Dzhaparidze, 1999) according to the formula: according to the formula: PIC = (2M + MM + MO): 4, where: M – mother's productivity; MO - the productivity of the father's mother; MM is the productivity of the mother's mother. Realization of the genetic potential (RGP) was determined by the formula: RGP = actual productivity/expected productivity according to PIC x100%.

To study the variability and heritability of milk production traits for the first lactation, mother-daughter pairs were selected. Each sample consisted of 47 cows and 47 offspring of German breed (Society of Limited Liability “Dastocom”), 110 cows and 110 offspring of French breed (Society of Limited Liability “Holstein”). The milk yield for 305 days of lactation, the fat content in milk and the amount of milk fat were studied, the variability of these indicators (Cv) was determined. The correlation coefficient (r) was calculated on a computer using the CORREL function in the Excel program environment. The heritability coefficient was found to be equal to twice the correlation coefficient between mothers and daughters (h^2 = 2rM/F). Student's t-test (t-test) was used to determine the significant difference between the data.

RESULTS AND DISCUSSIONS

Research in both herds began with a study of the productivity of the female ancestors of imported heifers. In Society of Limited Liability “Dastocom” pregnant heifers were brought from Germany.

The milk yield of mothers of fathers according to the highest finished lactation in Society of Limited Liability “Dastocom” (Table 1), averaged 12636 kg of milk, fat content 3.93%, amount of milk fat - 497 kg.

Table 1. Productivity of female ancestors of Holstein cows of German breed, Society of Limited Liability “Dastocom”

Indicators	Number of cows, heads	Milk yield, kg		Fat content, %		Amount of milk fat, kg	
		X ± Sx	Cv, %	X ± Sx	Cv, %	X ± Sx	Cv, %
Mothers	47	9832±181.7	17.9	3.86±0.05	10.3	379±7.5	17.5
Father's mother	45	12636±355.1	18.5	3.93±0.07	13.1	497±17.3	17.7
Mothers of mothers	45	10025±282.9	16.7	3.88±0.07	11.1	390±15.0	16.9

The milk yield of mothers of pregnant heifers on average per lactation amounted to 9832 kg of milk, the fat content was 3.86%.

The coefficients of variability in milk yield for all analyzed groups of mothers in the population of Society of Limited Liability “Dastocom” were slightly lower than the literature data and varied within 16.7% (mothers of mothers) and 18.5% (mothers of fathers), which characterizes a high concentration of female ancestors for this sign. According to the content of fat in milk, all female ancestors have a high coefficient of variability.

The results of the study of the milk productivity of the female ancestors of the Holstein breed, imported pregnant heifers from France (breeding farm Society of Limited Liability "Holstein"), showed that the average milk yield of mothers was 8413 kg of milk with a fat content of 3.75% (Table 2).

Table 2. Productivity of female ancestors of Holstein cows of French breed, Society of Limited Liability "Holstein"

Indicators	Number of cows, heads	Milk yield. kg		Fat content, %		Amount of milk fat, kg	
		X ± Sx	Cv, %	X±Sx	Cv, %	X ± Sx	Cv, %
Mothers	120	8413±167.1	27.6	3.75±0.05	14.5	312±6.5	22.8
Father's mother	138	11215±189.9	27.6	3.92±0.05	27.6	440±9.7	25.8
Mothers of mothers	125	9299±141.6	27.6	3.79±0.04	12.4	350±5.9	18.8

The milk yield of mothers of fathers for the highest lactation averaged 11215 kg of milk with a fat content of 3.92%, the amount of milk fat was 440 kg.
The coefficients of variability (Cv) for milk yield were the same for all analyzed groups of mothers - 27.6%. In terms of the content and amount of milk fat, the values of this trait on average for the sample were high and varied within 14.5-27.6% and 18.8-25.8%, respectively. Consequently, the imported population of French Holstein cattle has a large range of variability and high genetic diversity.
The results of the study of the milk productivity of the population of cows of the Holstein breed of the German selection of the breeding farm Society of Limited Liability "Dastocom" for the first two completed lactations are shown in Table 3.

Table 3. Dynamics of milk productivity of cows of Society of Limited Liability "Dastocom"

No	Indicators	The first lactation		The second lactation		Population average	
		X ± Sx	Cv, %	X ± Sx	Cv, %	X ± Sx	Cv, %
1.	Number of cows, heads	47		23		70	
2.	Milk yield, kg	8851 ± 81.5	6.3	9508 ± 211.0*	10.6	9067 ± 95.0	8.8
3.	Fat content, %	3.91 ± 0.01	1.1	3.95 ± 0.01	1.4	3.92 ± 0.01	1.3
4.	Amount of milk fat, kg	346 ± 3.1	6.1	358 ± 17.9	24.0	350 ± 6.2	14.8

Note: * - $P<0.05$

It was established that the milk yield of cows for the first lactation averaged 8851 kg of milk. An analysis of the milk yield of cows in the dynamics of lactations showed that during the second lactation, milk productivity was by 651 kg more than in the first lactation, the difference is significant at $P<0.05$.
The coefficient of variability (Cv) for milk yield per lactation was lower than the literature data and fluctuated within 6.3% and 10.6%, respectively, the first-second lactation, or an increase trend is observed in the dynamics of lactations. Low rates of variability characterize the high consolidation of animals in the development of the trait and reduce the possibility of selection when breeding them in a closed population. It should be noted that, on average, for the sample, the coefficients for milk yield, fat content in milk and the amount of milk fat amounted to 8.8%, 1.3% and 14.8%, respectively, which indicates good selection opportunities for the studied traits and is confirmed by the results of a comparative analysis of the level of milk productivity with the requirements of the standard for the Holstein breed (Figure 1).

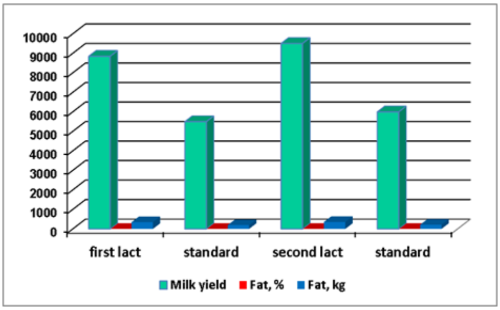

Figure 1. Comparative assessment of the milk productivity of cows of Society of Limited Liability "Dastocom" with the standard of the Holstein breed

Thus, the milk yields of cows for the to the first lactation compared to the breed standard were by 3351 kg more milk, the fat content - 0.31% and the amount of fat - 148 kg, for the second lactation the excess was by 3508 kg, 0.35% and 142 kg, respectively.

As a result of studying the productive qualities of cows of the Holstein breed of French selection (Society of Limited Liability “Holstein”), it was found that the milk yield of the analyzed number of cows increased with each subsequent lactation (Table 4).

Table 4. Characteristics of cows of the Holstein breed of French selection in terms of milk production for 305 days of lactation, Society of Limited Liability “Holstein” (X ± Sx)

Lactation	Number of cows, n	Indicators					
		Milk yield, kg	Cv, %	Fat content, %	C_v, %	The amount of milk fat, kg	C_v, %
the first	152	6334±34.7	6.8	3.97±0.004	1.1	252±1.3	6.5
the second	144	6547±31.8***	5.8	3.98±0.004	1.1	276±16.3	71
the third	97	6641±46.7***	6.9	3.97±0.005	1.2	264±1.7	27.6
the fourth	97	6881±40.2***	5.8	3.94±0.005	1.1	271±1.5	5.3
the fifth	64	6649±51.9***	6.2	3.95±0.005	1.1	262±1.9	27.6

Note: *** - $P < 0.001$.

Thus, if on the first lactation the cows produced an average of 6334 kg of milk, then on the second, third and fourth lactation they were by 213, 307 and 547 kg of milk more, respectively, the difference is significant at P<0.001. Milk yields on average for the fifth lactation decreased slightly compared to the previous three lactations, however, compared to the first lactation, the yields were by 315 kg of more milk, a significant difference P<0.001. Thus, the milk yields of the cows of the herd of Society of Limited Liability “Holstein” in the dynamics of lactation gradually increased including the fourth lactation, which is clearly shown in the figure of the diagram (Figure 2).

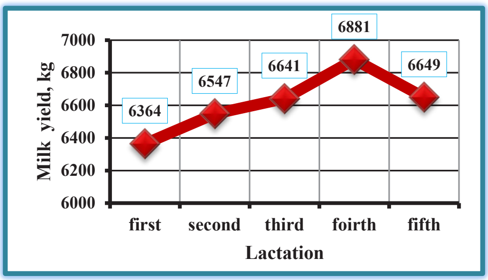

Figure 2. Milk productivity of cows of the breeding farm Society of Limited Liability “Holstein” in the dynamics of lactation

It should be noted that, depending on lactation, the lowest coefficient of variability in milk yield per lactation was at cows in the second and third lactations - 6.9%. The same is observed for the content of milk fat in milk, the coefficients of variability did not correspond and were lower than the literature data.

In terms of the amount of milk fat, the coefficient of variability for the first (6.5%) and fourth (5.3%) lactations was low. For other lactations, it was within the normal range (third and fifth lactations - 27.6%), or above the norm by 53-39%. Thus, in the population of Holstein cattle of French selection, the genetic diversity is reduced, an indirect expression of this is the low value of the coefficients of variability for all analyzed indicators.

One of the factors determining the value of imported livestock is the genetic potential of the acquired animals. As it is known, the genetic potential is determined by the productivity of maternal ancestors. In order to assess the potential capabilities of animals for all indicators of female ancestors in the analyzed populations, upon completion of their first lactation, the parental index of cows was calculated, which shows the genetic capabilities of the animal and the degree of transmission of productive qualities to offspring. For this, the parental index of cows was calculated, as well as the indicator of the realization of the genetic potential, the results are shown in Tables 5-6.

As it can be seen from the data in Table 5, the parental index of cows of Society of Limited Liability “Dastocom” for milk yield was 10581 kg of milk at primiparous cows. The implementation of the genetic potential for milk yield for 305 days of lactation was at the level of 83.6%, for fat - 100.7%.

Table 5. Realization of the genetic potential of cows in 305 days of the first lactation, Society of Limited Liability "Dastocom"

Indicators					
Parent index of cows (PIC)		Own productivity		Realization of genetic potential (RGP), %	
Milk yield, kg M ± m	Fat, % M ± m	Milk yield, kg M ± m	Fat, % M ± m	Milk yield	Fat
10581 ± 250.4	3.88 ± 0.06	8851 ± 81.5	3.91 ± 0.01	83.6	100.7

Table 6. Realization of the genetic potential of primiparous cows for 305 days of lactation, Society of Limited Liability "Holstein"

Indicators					
Parent index of cows (PIC)		Own productivity		Realization of genetic potential (RGP), %	
Milk yield, kg M ± m	Fat, % M ± m	Milk yield, kg M ± m	Fat, % M ± m	Milk yield	Fat
9335 ± 166.4	3.86 ± 0.09	6334 ± 34.7	3.97 ± 0.004	67.8	102.8

It was established that the parental index of cows in the population of the herd of Society of Limited Liability "Holstein" was at the level of 9335 kg in terms of milk yield, and in terms of fat content - 3.86%. The implementation of the genetic potential for milk yield was 67.8%, for the fat content in milk it was 102.8%. As is known, the correlation between the amount of milk yield and the content of fat in milk depends on many factors, including between the signs of the productivity of daughters and their maternal ancestors.

As it is known, most of the traits by which cattle's breeding is carried out are interconnected. Communication can be expressed only by a correlation relation. The results of the study of the relationship between individual indicators of productivity at cows of Society of Limited Liability "Holstein" depending on lactation are shown in Table 7.

Table 7. Correlation between productivity indicators, r ± m (Society of Limited Liability "Holstein")

Indicators	First lactation	Second lactation	Third lactation	Fourth lactation	Fifth lactation
Milk yield (X) - Fat, % (Y)	-0.397 ± 0.07	-0.617 ± 0.06*	-0.696 ±0.05***	-0.489 ± 0.08	-0.438 ± 0.01
Milk yield (X) - Fat, kg (Y)	+0.972 ± 0.02	+0.0669 ± 0.08	+0.990 ± 0.002	+0.982 ± 0.04	+0.987 ±0.003

Note: * - P < 0.05; *** - P < 0.001

The relationship between milk yield and the percentage of fat in milk of cows of the first to fifth lactation was in a negative correlation from moderate (-0.397, first lactation, -0.438 - fifth lactation), to noticeable - (-0.617, second lactation) and high (-0.696, third lactation). A comparative analysis of the results of the relationship between milk yield and percentage of fat it is found a significant difference between the third and first lactations - 0.299 at P <0.001, and between the second and first lactations - 0.220 at P<0.05.

As it can be seen, one-sided selection for the level of milk yield in the herd of Society of Limited Liability "Holstein" led to an increase of the negative relationship between these traits, which makes it difficult to conduct successful selection and indicates the need for simultaneous selection for milk yield and fat content in milk.

The nature of the dependence of the productive qualities of daughters and their mothers on the first lactation in the livestock population of Society of Limited Liability "Dastocom" was studied, the results of the studies are given in Table 8.

A negative relationship was established for milk yield for 305 days of lactation between mothers and their daughters (-0.378), the tightness of the relationship is moderate. According to the fat content, the correlation is positive, the closeness of the relationship is weak (+0.140).

Table 8. Correlation between the main productivity indicators of mothers and their daughters (Society of Limited Liability "Dastocom"), $r \pm m_r$

Indicators	Livestock mothers-daughters, n	Milk yield, kg	Fat content, %	The amount of milk fat, kg
Mothers - daughters	47-47	-0.378±0.01	+0.140±0.1	-0.143±0.1

A negative relationship was established for milk yield for 305 days of lactation between mothers and their daughters (-0.378), the tightness of the relationship is moderate. According to the fat content, the correlation is positive, the closeness of the relationship is weak (+0.140).

For assessment of the indicators of heritability, the method of correlation of the productivity indicators of daughters and their mothers was used, the results obtained are shown in Table 9.

Table 9. Heritability (h^2) of productivity indicators (mother-daughter) of the Holstein breed, Society of Limited Liability "Dastocom"

Indicators	Livestock mother-daughter, n	$h^2=2r_{m/f}$	$h^2=2r_{m/f}$, %
By milk yield	47	0.756	75.6
By fat content in milk	47	0.280	28.0
According to the amount of milk fat	47	0.286	28.6

As a result of the analysis, was revealed a high level of heritability for milk yield (mother-daughter) at cattle of the Holstein breed of German selection, which amounted to 0.756. It should be noted that the milk yield of mother cows is 75.6% due to heredity, which was inherited by their daughters. The content of fat in milk (h^2 = 0.280) was more affected by paratypic factors, as the heritability coefficients were quite low.

Of particular interest for the breeding process is the study of the dependence of the productive qualities of the offspring and its closest ancestors - M (mother), FM (father's mother) and MM (mother's mother). The results of the study of the dependence of productivity indicators between maternal ancestors and daughters in the herd of Society of Limited Liability "Dastocom" are shown in Table 10.

Table 10. Correlation of the main productive indicators of the offspring and its closest ancestors, $r \pm m_r$

Indicators	Daughter - Mother (M)	Daughter - mother's mother (MM)	Daughter - father's mother (FM)
Milk yield, kg	-0.378 ± 0.14***	-0.247 ± 0.15	-0.047 ± 0.15
Fat, %	+0.280 ± 0.14	+0.194 ± 0.15	-0.124 ± 0.15
Fat, kg	-0.140 ± 0.15	-0.026 ± 0.15	-0.111 ± 0.15

Note: *** - P<0.001.

As it can be seen by milk yield between maternal ancestors (M) and daughters, a negative moderate relationship was revealed – (-0.3780 ± 0.14). Between paternal ancestors (FM) and daughters, the correlation by milk yield is weakly negative (-0.047 ± 0.15).

According to the content of fat in milk, the relationship between maternal ancestors is weakly positive +0.280 (M) and +0.194 (MM), between paternal ancestors (MO) - weakly negative -0.124. A comparative analysis of the relationship between the group of daughters of paternal ancestors (FM) and maternal ancestors (M) revealed the superiority of maternal ancestors at r= -0.331, the difference is significant (P<0.001).

Therefore, when selecting animals in the herd of Society of Limited Liability "Dastocom" it is necessary to attach great importance to milk yield and fat content in the milk of maternal ancestors.

The effectiveness of the selection cattle on productivity is determined by the degree of hereditary improvement of each new generation compared to the previous one. The results of the study of the share of the influence of genetic factors on the productive qualities of primiparous cows in the herd of Society of Limited Liability "Dastocom" are shown in Table 11.

Table 11. Heritability of the main productive features at primiparous cows, h^2 (Society of Limited Liability "Dastocom")

No	Indicators	Daughters - Mothers (M)	Daughter - mother's mother (MM)	Daughter - father's mother (FM)
1.	Milk yield, kg	0.76	0.10	0.49
2.	Fat, %	0.28	0.25	0.39
3.	Fat, kg	0.29	0.22	0.05

As it can be seen, the heritability of the size of the milk yield of analyzed animals is higher than the content of fat in milk and the amount of milk fat. Mothers had the greatest influence on milk yield - on average, the heritability coefficient h^2 = 0.76, paternal ancestors (father's mother) had a slightly lesser influence - h^2=0.49. The fat content in the milk of primiparous cows was largely due to heredity, where paternal ancestors h^2 = 0.39 had the greatest influence. Thus, a high coefficient of heritability for milk yield indicates the uniformity and stability of the conditions for keeping and feeding animals in the herd of Society of Limited Liability "Dastocom".

The results of the study of the share of the influence of genetic factors on the productive qualities of primiparous cows, and also the correlation between the main indicators of the productivity of cows and their maternal ancestors in the herd of Society of Limited Liability "Holstein" are shown in Table 12.

Table 12. Correlation and heritability of the main productive traits of daughters and close ancestors, Society of Limited Liability "Holstein"

No	Indicators		Daughter - Mother (M)	Daughter - mother's mother (MM)	Daughter - father's mother (FM)
1.	Milk yield	r	+0.119 ± 0.08	+0.037 ± 0.08	+0.247 ± 0.07
		h^2	0.24	0.074	0.49
2.	Fat	r	+0.219 ± 0.08	+0.024 ± 0.08	-0.159 ± 0.08
		h^2	0.44	0.048	0.32

As it can be seen from the data in Table 12, the correlation on the milk yield of daughters and close female ancestors is positive, the tightness of the relationship is weak, the greatest relationship was found between paternal ancestors (FM) - (r = +0.247). According to the content of fat in milk, the correlation between daughters and mothers was (r = +0.219).

Based on the obtained results, presented in Table 12, it can be concluded that for the entire population, milk yield and fat content in the milk of primiparous cows, was largely due to heredity (h^2 = 0.24-0.49) and (h^2 = 0.44-0.32), respectively.

Paternal ancestors had the greatest influence on milk yield - the average heritability coefficient was 0.49. And the maternal ancestors had the greatest influence on the fat content, in which the heritability coefficient was h^2 = 0.44, and paternal ancestors had a slightly lesser influence - h^2 = 0.32. Thus, the realization of the genetic potential of the heifers of Society of Limited Liability "Holstein" was mainly influenced by genetic factors.

CONCLUSIONS

1. Analysis of the milk yield of cows of the Holstein breed of German selection (Society of Limited Liability "Dastocom') in the dynamics of lactation showed that during the second lactation, milk productivity was by 651 kg of milk more than during the first lactation, the difference is significant at $P<0.05$.

2. It was established that the milk yield of cows of the Holstein breed of French selection (Society of Limited Liability "Holstein") increased with each subsequent lactation. So, if in the first lactation milk yields averaged 6334 kg of milk, then in the second, third and fourth

lactations they were 213, 307 and 547 kg of milk more, respectively, the difference is significant at $P<0.001$.

3. The relationship between milk yield and percentage of fat in milk of cows of the first - fifth lactation in the herd (Society of Limited Liability "Holstein") was in a negative correlation from moderate (-0.397, first lactation, -0.438 - fifth lactation), to noticeable - (-0.617, second lactation) and high (-0.696, third lactation).

4. At cattle of the Holstein breed of German selection (Society of Limited Liability "Dastocom"), a high level of heritability for milk yield (mother-daughter) was found, which amounted to 0.756. The milk yield of cows-mothers is 75.6% due to heredity, which was inherited by their daughters. The content of fat in milk (h^2 = 0.280) was more affected by paratypic factors, since the heritability coefficients were quite low.

5. The heritability of milk yield magnitude at primiparous cows of Society of Limited Liability "Dastocom" is higher than the fat content in milk and the amount of milk fat. Mothers had the greatest influence on milk yields - on average, the heritability coefficient h^2 = 0.76, paternal ancestors (father's mother) had a slightly lesser influence - h^2 = 0.49. The fat content in the milk of primiparous cows was largely due to heredity, where paternal ancestors had the greatest influence h^2=0.39.

6. For the entire analyzed population of primiparous cows of Society of Limited Liability "Holstein", milk yield and fat content in milk were largely determined by heredity (h^2 = 0.24-0.49) and (h^2 = 0.44-0.32), respectively. Paternal ancestors had the greatest influence on milk yield - the average heritability coefficient was 0.49. The maternal ancestors had the greatest influence on the fat content, at which the heritability coefficient was h^2 = 0.44, and the paternal ancestors had a slightly lesser influence - h^2 = 0.32.

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EVALUATION OF STUD BULLS BY BETA-CASEIN GENOTYPE IN THE CONTEXT OF CONSERVATION OF LOCAL CATTLE BREEDS IN UKRAINE

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Abstract

Genotyping of 114 stud bulls of local and specialized dairy breeds based on the beta-casein gene has been carried out. According to the results of the research, it is found that the highest frequency of the A2A2 desired homozygous genotype is characteristic of local cattle of Lebedyn breed and OBV (56%), as well as stud bulls of the Ukrainian White Headed breed (50). A significant difference in the frequency of genotypes for this trait has been established between individual breeds. The creation of micro-populations of cattle based on beta-casein with the A2A2 desired homozygous genotype makes it possible to obtain milk that has a number of properties, which are not characteristic of ordinary milk. Thus, the increased frequency of the A2A2 genotype for beta-casein may contribute to the conservation and spread of local breeds on Ukrainian farms.

Key words*: allele, beta-casein, breed, genotype, stud bull.*

INTRODUCTION

In accordance with international requirements, in 2000 Ukraine developed the first National Program for the Conservation and Rational Use of the Breed Gene Pool. An important aspect of this program is the genetic evaluation of stud bulls of local breeds (Kruhliak, 2017).
Scientists claim that in Ukraine in the period up to 2014, 16 domestic breeds and breed groups disappeared only from the mammalian class, and this is, first of all, the disappearance of valuable genes that characterized adaptability to the conditions of the environment in which cattle existed, resistance to adverse factors, and guaranteed product quality. These genes will not be able to be restored in a number of generations (Reznykova, 2022).
It is believed that breeds, if desired, can be revived and expanded by both natural and artificial reproduction methods. For this purpose, the Bank of Genetic Resources of M.V. Zubets Institute of Animal Breeding and Genetics of the National Academy of Agrarian Sciences has created a sufficient supply of semen of stud bulls in a deep frozen state. It should be added that the semen reserves of stud bulls of individual local breeds are also stored in other semen banks of breeding enterprises in Ukraine that enables to use such reserves for the revival of endangered populations. Theoretically, it is not a significant problem to revive the number of Brown cattle breeds, even in the absence of breeding farms and breeding stock of the active part of the population. Cows of this breed can be found in the population, or absorption crossing may be used if the animal is not purebred (Vyshnevskyi, 2017).
At the present stage, the main purpose of the bank is to accumulate and ensure long-term storage of gene pool material of all types of farm animals, as well as to implement a set of organizational and technological measures for

the preservation and rational use of their gene pool in Ukraine (Baschenko et al., 2017).
It is generally known that the progress of breed in modern breeding conditions is provided by stud bulls with high breeding value. This has become possible by storing deep-frozen semen and applying a large-scale breeding system in practice (Ladyka et al., 2019).
To implement the Gene Pool Preservation Program, the bank of genetic resources of animals stores and uses genetic material from 27 stud bulls of three local breeds - Ukrainian White Headed, Lebedyn and Brown Carpathian. Semen of the Red Steppe breed from two stud bulls is stored in the gene pool bank. Scientists state that to characterize the biological diversity of genetic material stored in the Institute's bank, it is expedient to analyze stud bulls by genealogical origin. This study should be combined with their molecular genetic characteristics. It is believed that an increase in the genetic potential of cattle is mainly determined by the availability of information on genes that control signs of productivity and enable to ensure the targeted selection of animals (Ladyka et al., 2019).
Much attention in dairy cattle breeding is paid to the quality characteristics of milk. In recent years, scientists have found that cow milk usually contains two main types of beta-casein, such as A1 and A2 (Gorkhali et al., 2021; Ivankovic et al., 2021; Park & Haenlen, 2021).
Researchers have found a possible relationship between milk consumption and certain diseases, such as Type 1 diabetes, cardiovascular disease, Sudden Infant Death Syndrome, schizophrenia and autism, gastrointestinal diseases, prostate cancer, and other diseases (Amatya et al., 2021; Gorkhali et al., 2020; Mumtaz et al., 2021).
The frequency of the A2/A2 genotype in Holstein cattle is 48%, A1/A2 heterozygotes are amounted to 25%, and A1/A1 homozygotes are amounted to 27%. This frequency in Ayrshire stud bulls is 22%, 47%, and 31%, respectively. At the same time, A. Parashar shows that the frequency of the A1 allele in the Guernsey breed is in the range of 4-2%, Swiss - 34-30%, Jersey - 50-37%, Holstein - 56-47%, Ayrshire - 60-51%, Red Danish - 77% (Parashar & Saini, 2015). Thus, DNA monitoring of the ratio of beta-casein alleles in the genotype of stud bulls will enable to predict the possibility of creating dairy herds with the programmed milk quality, since an increase in beta-casein homozygosity of A2A2 in the next generation is possible, especially when using A2A2 homozygous stud bulls. This, in turn, will increase the competitiveness of local breeds, and accordingly give additional options for their preservation.

MATERIALS AND METHODS

The research was conducted at the premises of the Laboratory of O.O. Bogomoletz Institute of Physiology of the National Academy of Sciences of Ukraine. The semen of stud bulls of the Ukrainian White Headed (n = 8), Ukrainian Gray (n=11), Red Steppe (n = 2) breeds stored in the Bank of Genetic Resources of M.V. Zubets Institute of Animal Breeding and Genetics of the National Academy of Agrarian Sciences, Lebedyn (n = 12) and Ukrainian Black-and-White dairy (n = 30), Simmental (n = 13) breeds of the semen bank of Sumy Breeding Center, Holstein (n = 15) breed of the semen bank of the Ukrainian Genetic Company was examined and studied. In addition, blood samples from the raised stud bulls of Lebedyn and OBV crossbreeds were examined (n=23).
Blood samples were taken under the sterile conditions into 2.7 mL Monovette contains EDTA potassium salt as an anticoagulant ("Sarstedt", Germany) with the following freezing of samples and their storage at -20ºC. DNA for genotyping was extracted from the samples using Monarch® Genomic DNA Purification Kit New England BioLab kits (USA) according to manufacturer's protocol. TaqMan®Custom was used to perform allelic discrimination. The TaqMan® SNP Genotyping Assays use TaqMan® 5′-nuclease chemistry for amplifying and detecting specific polymer-phisms in purified genomic DNA samples. All assays are developed using Life Technologies robust bioinformatics assay design process relying on a pipeline using heuristic rules deduced from both manufacturing and assay performance data. The TagMan@Genotyping system and a set of primers and probes were used to perform allelic discrimination.
SNP rs43703011 in the beta-casein gene (CSN2) was determined according to our methods using the next primers: upstream 5'-CCCAGACACAGTCTCTAGTCTATCC-3',

downstream 5’-GGTTTGAGTAAGAGGAGGGATGTTT-3’ and the next probe - 5’-VIC-CCCATCCATAACAGCC-MGB-3` and 5’-FAM-CCATCCCTAACAGCC-MGB-3` (Thermo Scientific, USA). Amplification realized using Fast Real-time PCR System (Applied Biosystems, США) in total volume 10 мкл with 2X TaqMan Universal Master Mix (Applied Biosystems, USA), primers, probes, and DNA. Amplification of 84 bp fragment of CSN2 consisted of two steps: denaturation (95°C) during 3 s and annealing and elongation (60°C) during 30 s. Data were analyzed using 7500 Fast Real-Time PCR Software.

The data analysis was performed in the R statistical environment (www.R-project.org, V.4.0) and STATISTICA 10.

The allele frequency was calculated taking into account the number of homozygotes and heterozygotes found in the corresponding allele using the following formula:

$$P(A) = \frac{2N_1+N_2}{2n} \quad (1)$$

where:

N_1 and N_2 - number of homozygotes and heterozygotes for the studied allele, respectively;

n - sample number.

In order to assess the statistical reliability of the discrepancy between the distribution of the obtained results the Pearson criterion was used:

$$X^2 = \frac{\Sigma(A-T)^2}{T} \quad (2)$$

where:

A - actual number of genotypes;

T - theoretical number of genotypes.

The actual (available) heterozygosity was determined by direct calculation using the following formula:

$$H_0 = \frac{N_2}{n} \quad (3)$$

The expected heterozygosity was determined using the following formula:

$$H_E = 1 - \sum_{I=1}^{n} p_i^2 \quad (4)$$

where:

$p_1, p_2, \ldots p_n$ - frequency of alleles.

The fixation index was calculated using the following formula:

$$F_{is} = \frac{H_E - H_0}{H_E} \quad (5)$$

RESULTS AND DISCUSSIONS

The results of DNA testing of the beta-casein locus for A1 and A2-allelic variants in stud bulls of the studied breeds and cross cattle have shown that the highest frequency of the A2A2 desired homozygous genotype is characteristic of the Lebedyn and OBV crossbreeds. They were somewhat inferior to stud bulls of the Ukrainian White Headed (Table 1).

It should be noted that this genotype was found in all local breeds studied, with the exception of two stud bulls of the Red Steppe breed characterized by the A1A1 and A1A2 genotypes (not shown in Table). In commercial breeds, Simmental cattle had a higher frequency of the desired genotype. Stud bulls of the Holstein breed had the lowest frequency of the A2A2 genotype.

Values of the frequency of the A2A2 genotype in stud bulls of the Ukrainian Gray and Lebedyn breeds which are slightly lower than the expected ones, in our opinion, require a more detailed study. Stud bulls of the Ukrainian Black-and-White dairy breed had a mediocre value of the frequency of the desired genotype.

Stud bulls of the Brown Carpathian and Holstein breeds are characterized by a greater frequency of the A1A2 homozygous genotype. The least frequency is found in stud bulls of the Ukrainian White Headed and Simmental breeds.

Stud bulls of the Ukrainian Gray breed have the highest frequency of the A1A1 genotype. These genotypes are not found in stud bulls of the Brown Carpathian breed and Lebedyn crossbreeds with OBV.

The local Lebedyn crossbreeds with OBV were dominated by frequency of the desired A2 allele. Cattle of Ukrainian White Headed, Simmental of the Austrian breeding and Brown Carpathian breeds have values close to the above. An interesting fact is the predominance of the frequency of this allele over the A1 allele in stud bulls of Holstein and Ukrainian Black-and-White dairy breeds. The predominance of the A1 allele in cattle of the Ukrainian Gray and Lebedyn breeds requires a more detailed study.

Table 1. Frequency of alleles and genotypes by the beta-casein gene locus in stud bulls

Breed/crossbreed	Distribution	Frequency of genotypes			Frequency of alleles		$\chi 2$
		A2A2	A1A2	A2A2	A1	A2	
Ukrainian White Headed	A	12.5	37.5	50.0	31.25	68.75	0.1295
	E	9.8	43.0	47.2			
Ukrainian Gray	A	36.4	45.5	18.1	59.10	40.90	0.0393
	E	34.9	48.3	16.8			
Lebedyn purebred	A	25.0	58.3	16.7	54.20	45.80	0.3667
	E	29.3	49.7	21.0			
Brown Carpathian	A	0.0	70.0	30.0	35.00	65.00	2.8994
	E	12.3	45.5	42.2			
Crossbreeds of Lebedyn breed with original German Brown breed	A	0.0	43.5	56.5	21.7	78.3	1.7746
	E	4.7	34.0	61.3			
Ukrainian Black-and-White dairy	A	26.7	43.3	30.0	48.3	51.7	0.5256
	E	23.4	49.9	26.7			
Holstein	A	6.7	73.3	20.0	43.0	57.0	3.6488
	E	18.8	49.1	32.1			
Simmental	A	15.4	38.5	46.1	34.6	65.4	0.2938
	E	12.0	45.2	42.8			

Source: Own research.

There is a generally accepted view that a violation of random crossing should cause a deviation in genotype frequencies from the expected equilibrium according to the Hardy-Weinberg principle. In cattle of the Lebedyn and Brown Carpathian breeds, crossbreeds of the Lebedyn and Original Brown and Holstein breeds, the actual heterozygosity exceeds the expected one. A negative value of the Wright Fixation Index indicates a slight excess of heterozygotes in these samples (Table 2). In our opinion, measures to preserve the Lebedyn breed developed by specialists of Sumy National Agrarian University (Ladyka et al., 2021; Ladyka et al., 2019), namely, the work by the population method of reciprocal reproduction using stud bulls of the Original Brown German breed, enables to increase the frequency of the A2 beta-casein allele in gene pool herds (according to our research, the frequency of the desired allele in stud bulls increased from 45.8% to 78.3%).

This, in turn, has made it possible to obtain cattle with the desired quality indicators of dairy raw materials and a number of stud bulls of the Lebedyn breed with the A2A2 beta-casein genotype for custom mating.

Table 2. Values of the main indicators of variability by the kappa-casein gene

Breed	H_o	H_e	F_{is}
Ukrainian White Headed	0.375	0.430	0.128
Ukrainian Gray	0.455	0.483	0.060
Lebedyn	0.583	0.497	-0.175
Brown Carpathian	0.700	0.455	-0.538
Crossbreeds of Lebedyn breed with original German Brown	0.435	0.340	-0.278
Ukrainian Black-and-White dairy	0.433	0.499	0.132
Holstein	0.733	0.491	-0.493
Simmental	0.385	0.453	0.150

Ho - actual heterozygosity, He - expected heterozygosity, Fis - fixation index

Source: Own research.

Therefore, we can say with confidence that due to the research and cooperation of scientists and producers, it is possible to create dairy herds of Brown cattle in order to obtain milk with the A2A2 genotype for beta-casein that will significantly increase the profitability of its production. In turn, this may contribute to further measures to preserve the gene pool of Brown cattle in Ukraine. It is also promising to use stud bulls of other (local) breeds in order to further preserve them and create herds of cattle with the A2A2 genotype.

Differentiation of the frequency of genotypes by beta-casein in cows of autochthonous breeds is established based on the results of studies that have been previously conducted (Table 3).

Cattle of the Lebedyn breed are characterized by a higher frequency of the desired A2A2 genotype, which confirms our opinion about the prospects of creating herds of cattle with the A2A2 genotype of this particular breed. Cattle of the Ukrainian Gray breed being the parent one in the creation of the Lebedyn breed have a low frequency of the A2A2 genotype - 0.046. At the same time, they are characterized by a high frequency of heterozygous genotypes that creates conditions for obtaining cattle of the desired genotype subject to the use of stud bulls with the A2A2 genotype. As our studies have shown, the share of such stud bulls whose semen is stored in a deep frozen state is equal to 18.2 %. Cows of the Ukrainian White Headed breed have a share of heterozygous genotype at the level of 52%, while 50% of stud bulls have the desired homozygous genotype that enables to work on creating herds of cattle with the A2A2 genotype.

The similar work with the Brown Carpathian cattle will be longer, which is due to the absence of cows with the A2A2 and A1A2 genotypes and a low proportion of stud bulls with the A2A2 genotype.

Table 3. Frequency of beta-casein genotypes in cows of local cattle breeds

Breed	Share of genotypes	Author
Lebedyn	A1A1-0; A1A2-0.7; A2A2-0.3	Ladyka V., Pavlenko Y., Sklyarenko Y., 2021
Ukrainian Gray	A1A1-0.110; A1A2-0.844; A2A2-0.046	Mokhnacheva N. B., 2021
Ukrainian White Headed	A1A1-0.48; A1A2-0.52; A2A2-0	Mokhnacheva N. B., 2021
Brown Carpathian	A1A1-1.00; A1A2-0; A2A2-0	Mokhnacheva N. B., 2021

Source: Links to other studies

CONCLUSIONS

This work results in finding frequencies of alleles and genotypes by the beta-casein locus in stud bulls of autochthonous breeds of Ukraine. It is established that according to this feature, stud bulls of local Ukrainian breeds differ significantly from each other. The results obtained indicate that there is no targeted breeding work on creating herds with the A2/A2 genotype cattle. An exception is the breeding of the Brown cattle, as evidenced by the obtaining of stud bulls from custom mating, in which the frequency of the desired genotype is higher than 50%. Stud bulls of the Ukrainian White Headed breed are also characterized by a similar frequency. Therefore, it can be argued that there are prospects for creating such herds in the population of Lebedyn and Ukrainian White Headed cattle. Such work with dairy herds of the Ukrainian Gray, Brown Carpathian and Red Steppe breeds requires further in-depth study.

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ESTIMATION OF GENETIC PARAMETERS FOR SCRAPIE RESISTANCE IN LOCAL BREEDS OF SHEEP RAISED IN ROMANIA

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Abstract

In the European Union, animal breeding programs have been implemented to increase scrapie resistance in sheep. In addition to increasing animal resistance to an infectious disease, selection for pathogen resistance has the potential to lessen the transmission of the pathogen to offspring, particularly when the population under consideration may serve as the primary pathogen reservoir. Several sheep populations from Romania were used in this study: Tsurcana; Tsigae; Merinos; Cap Negru de Teleorman and some imported breeds: Suffolk; Ille de France and Awassi. Sanger sequencing method was used to identify PRNP genetic polymorphism, at 136, 154 and 171 codons. From the analyzed samples, a moderate share of the homozygous ARR allele, responsible for the highest resistance to scrapie, respectively (R1), was observed, and the heterozygous ARR/ARQ (R2) and homozygous ARQ/ARQ (R3) genotypes had an abundance of over 50% of the genotype panel. In practice, the use of phenotype as input to the model is given by numbers (risk classes from 1 to 5). To estimate the heritability of resistance to scrapie, the animal threshold model was used.

***Key words**: scrapie, genotype, sanger sequencing, trehold animal model, heritability.*

INTRODUCTION

Sheep and goats are susceptible to the transmissible spongiform encephalopathy (TSE) known as scrapie, which has a protracted incubation period and progressing neurological symptoms like tremors, pruritus, and ataxia (Jeffrey & Gonzalez, 2007). The disease is brought on by an accumulation of improperly folded prion protein in the brains of infected animals, which results in the death of neuronal cells and the development of sponge-like vacuoles (Mabbott, 2020). There is currently no cure or vaccine for scrapie, and the precise mechanisms underlying prion pathogenesis and transmission remain poorly understood.

The potential transmission pathways for scrapie have recently come to light and include direct contact with infected animals, contaminated environments, and vertical transmission from ewes to their offspring (Konold et al., 2008). Additionally, extensive research into the genetics of scrapie susceptibility in sheep led to the identification of a number of prion protein gene (PRNP) polymorphisms that are related to varying degrees of disease susceptibility (White et al., 2008) with the identification of several prion protein gene (PRNP) polymorphisms associated with varying degrees of disease susceptibility, genetic susceptibility to scrapie has been thoroughly investigated (Jeffrey & Gonzalez, 2007). Numerous studies have shown scrapie to be heritable, indicating that genetic factors significantly influence disease susceptibility (White et al., 2008).

According to genetic studies Otelea et al. (2011) consider that PRNP polymorphisms at codons 136, 154, and 171 were strongly associated with classical scrapie. When this polymorphism is combined, it results in five PRNP codon haplotypes or alleles and fifteen PRNP diploid genotype combinations found in sheep. The susceptibility of sheep to scrapie varies greatly between genotypes, ranging from high resistance (ARR/ARR) to severe vulnerability (VRQ/VRQ). The primary goal of this study is to estimate the heritability for scrapie resistance of rams accepted for reproduction and raised on Romanian territory, with a secondary goal of presenting a common method of screening for scrapie resistance.

MATERIALS AND METHODS

The method and materials used for DNA extraction, amplification of the segment of interest and genomic sequencing, is similar to the methods used by other researchers Otelea et al. (2011). For this study, 3918 samples belonging to different breeds raised in Romania were used. Blood samples were collected in 10 ml tubes containing K3-EDTA anticoagulant and were kept at -20 degrees Celsius until DNA extraction. Thermo Fischer Pure Link Genomic DNA mini Kit was used for DNA extraction. The obtained DNA was quantified to verify the quantity and purity using a Nanodrop One spectophotometer. The clasic PCR technique was used to amplify the DNA sequence,The PCR was set up in a volume of 25 µl with 2 µl DNA solution, the volume of reagents for 1 reaction: 0.125 µl Ampli GoTaq Polymerase-Promega (5U/µl), 1.5 µl MgCl2 (25 mM), 0.5 µl dNTP mix (10 mM), 5 µl buffer 10 x for polymerase, 0.6 µM of forward primer(5'-GGTCAAGGTGGTAGCCACAGTCAGTGG AAC-3') and 0.6µM of reverse primer (5'-ATCACCCAGTACCAGAGAGAATCCCAG GCT-3'). The thermal cycling program included: a denaturation (3 min at 95°C), 40 cycles of amplification (45 s at 95°C, 45 s at 59°C, 60 s at 72°C), and a final extension (7 min at 72°C). It was used an Thermal Cycler from Thermo Scientific. The electrophoresis migration method was used to check the quality and the lenght of the amplified segment. Agarose gel electrophoresis. PCR products (7 µl) were separated on a 10% agarose gel (70V for 45min) containing ethidium bromide in TBE buffer (10 mM Tris, 2.75 g boric acid/l, 1 mM Na2 EDTA). The visualization was performed in UV transilluminator (Bio-Rad) and the images were captured with a software from Bio-Rad (Otelea et al., 2011).

Sequencing: The PCR reaction products were purified with ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems™ Foster City, CA, USA). The DNA sequencing reactions were done using BigDye Terminator Kit v3.1 (Applied Biosystems). The precipitation of DNA sequencing product was performed with BigDye XTerminator® Purification Kit, (Applied Biosystems, Foster City, CA, USA). The primers used for sequencing were the same as for the PCR amplification. The sequencing was performed on 3300 Hitachi Genetic Analyzer (Applied Biosystems) (Otelea et al., 2011).

RESULTS AND DISCUSSIONS

After analyzing the samples from 2019 and 2020, it was observed that of all 15 possible combinations of alleles responsible for the degree of susceptibility to scrapie, only 13 were expressed, from the 2019 genotype table, the ARH/ARH homozygous and AHQ/ARH heterozygous genotypes are missing, and in the 2020 population, ARQ/ARH and ARQ/AHQ heterozygous genotypes are missing. The homozygous ARR/ARR allele was observed in 16.8% of the 2019 population with a slight upward trend in the 2020 population, respectively. 17.98%. A considerable similarity between the two populations would be related to the abundance of the heterozygous ARR/ARQ genotype, which occupies approximately half of the genotype panel (38.79% - 2019)/(40.30% - 2020). A considerable frequency of homozygous ARQ/ARQ genotype was also observed. ARR/ARH and ARQ/ARH have a low prevalence, and the other genotypes are found sporadically in populations (Figures1 and 2).

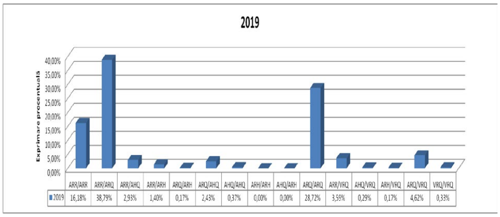

Figure 1. Distribution of genotypes
for the breeds tested in 2019

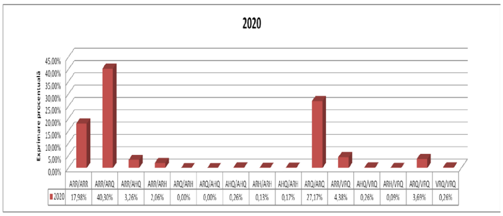

Figure 2. Distribution of genotypes
for the breeds tested in 2020

From the samples analyzed in 2021, a moderate distribution of the homozygous ARR allele (R1), responsible for the highest resistance to scrapie, was observed, and the heterozygous ARR/ARQ(R2) and homozygous RQ/ARQ(R3) genotypes had an abundance of over 50% of the panel of genotypes. Although the distribution of genotype frequencies seems similar to that of 2019 and 2020, in 2021 the share of breeds for testing was very small, implicitly the number of individuals tested was much smaller (Figure 3).

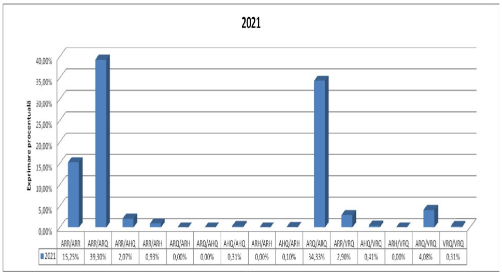

Figure 3. Distribution of genotypes for the breeds tested in 2021

In the context of scrapie susceptibility in sheep,we used the threshold animal modelto estimate the heritability of the trait by analyzing data from genotyping samples and measuring scrapie susceptibility in different animals.

The animal threshold model is a statistical approach used to estimate the heritability of scrapie in sheep, which assumes that there is a continuous underlying liability to the disease, with animals crossing a threshold to become clinically affected. This model estimates the proportion of variance in liability that is due to genetic factors, providing valuable insights into the genetic factors that influence disease susceptibility and resistance. Several studies have used the animal threshold model to estimate the heritability of scrapie in different sheep populations, with heritability estimates ranging from 0.28 to 0.44. While the animal threshold model has some limitations, it remains one of the most widely used methods for estimating the heritability of scrapie in sheep (Houston et al., 2003; Pálsson et al., 2004; Toppinen et al., 2008).

The model allows researchers to control for factors such as environmental factors that may influence scrapie susceptibility, and to estimate the genetic variance and covariance of the trait.

Since the classification of animals into risk classes - classes from (R1) to (R5) - does not have a normal distribution for estimating the heritability for the 7 sheep breeds, the animal threshold model was used. The model is composed of the incidence matrices and their associated vectors.

The obtained results revealed that sheep resistance to scrapie has a variable genetic determinism, from highly heritable (0.925 in the Tsurcana breed and 0.801 in the Merinos breed) to weakly heritable (0.563 Tsigae, 0.376 Cap Negru de Teleorman; 0.325 Suffolk; 0 .31 Ille de France and 0.194 Awassi) (Table 1).

Other study has investigated the heritability of scrapie susceptibility using genetic approaches such as quantitative trait locus (QTL) mapping and genome-wide association studies (GWAS). These studies aim to identify specific regions of the genome that are associated with scrapie resistance or susceptibility (Houston et al., 2003).

Moreno et al. (2010) observed that heritability of polygenic and QTL effects for log-transformed incubation time in different PrP populations is moderate, ranging from 61% (*p = 0.05*) to 74% (*p = 0.04*) at different significance levels.

$$\lambda_{ijk} = f(t)_i + H_j + a_k + e_{ijk}$$

Figure 4. Animal threshold model vectorial writing

$$\lambda = Ft + Xb + Zu + e$$

$$\begin{bmatrix} Q & L'X & L'Z \\ X'L & X'WX & X'WZ \\ Z'L & Z'WX & Z'WZ + G^{-1} \end{bmatrix} \begin{bmatrix} \Delta t \\ \Delta b \\ \Delta u \end{bmatrix} = \begin{bmatrix} p \\ X'v \\ Z'v - G^{-1}u \end{bmatrix}$$

Figure 5. Animal threshold model in matrix writing

Table 1. Estimated heritability for each breed using the threshold animal model

Breed	Observations	σ_a	σ_e	σ_t	h^2
Awassi	14	1.103	4.583	5.686	0.194
Ille de France	41	1.626	3.615	5.241	0.31
Suffolk	37	1.215	2.514	3.73	0.325
Cap Negru de Teleorman	106	3.679	6.096	9.775	0.376
Tsigaie	242	7.123	5.529	12.652	0.563
Merinos	822	21.873	5.428	27.3	0.801
Tsurcana	2656	68.587	5.552	74.14	0.925

σ_a-additivevarriance
σ_e- error of varriance
σ_t-totalvariance

Following the analysis of the obtained data in Table 1, it was observed that in the imported breeds: Ille de France, Awassi, Suffolk, even if the individuals were classified in resistant classes (R1); (R2), the weaker genetic determinism was also influenced by the number of genotyped individuals. This is due to the fact that larger populations are more likely to contain a broader range of genotypes, which can aid in capturing more genetic variation in the trait of interest.

If the population is small, individuals may have limited genetic variation, resulting in an underestimation of heritability. In contrast, a large population may have more genetic variation among individuals, resulting in a more accurate estimation of heritability.

CONCLUSIONS

The animal threshold model, by estimating the heritability of scrapie susceptibility, can help identify the genetic factors that contribute to the disease and can inform strategies for controlling and preventing the disease through selective breeding programs.

Overall, the animal threshold model is an effective tool for determining the genetic basis of complex traits like scrapie susceptibility in sheep, and it has been widely used in animal breeding and genetics research.

The animal threshold model has some drawbacks, such as the assumption of ongoing vulnerability to the disease and the requirement for precise clinical case diagnosis. But it is still one of the most popular techniques for determining the heritability of scrapie in sheep, and it has given us important knowledge about the genetics of disease susceptibility and resistance.

Sanger sequencing is a trustworthy method for identifying specific genetic mutations linked to scrapie resistance or susceptibility in sheep and goats. It can determine whether an animal has a genotype associated with a higher or lower risk of developing scrapie by sequencing specific regions of the prion protein gene. This data can be used to make informed breeding decisions and to develop targeted scrapie control programs in livestock populations.

To completely comprehend the intricate genetic and environmental components that contribute to scrapie susceptibility in sheep, more research is required.

Sanger sequencing can be an effective research technique, but to fully understand the underlying mechanisms, it should be used in conjunction with other methods like gene expression analyses and functional studies.

It is important to note that scrapie heritability is only one factor that influences the disease's occurrence. Environmental factors, such as feed contamination, can also have a significant impact.

As a result, both genetic and environmental factors should be considered in flock management and disease control.

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ASSOCIATION OF *FABP3* GENE POLYMORPHISM WITH LITTER SIZE IN EWES FROM THE BULGARIAN DAIRY SYNTHETIC POPULATION

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Abstract

The Bulgarian Dairy Synthetic Population (BDSP) is the most numerous breed in Bulgaria. FABP3 gene plays a crucial role in hormone action and cellular functions. The aim of the study is to investigate the relationship of FABP3 gene polymorphism with litter size in ewes of BDSP. This experiment involved 110 ewes from the herd of the Agricultural Institute-Shumen. Ewes were selected by birth type (singles, twins, triplets) and with records of the number of lambs born from a minimum of two consecutive lambing. The average number lambing of ewe is 3.94. Two alleles and two genotypes were identified in the studied animals in exon 2 of the FABP3 gene (SNP3) by the PCR-RFLP technique with BseDI endonuclease. The association of FABP3 gene polymorphism with total litter size and litter size depending on the parity and type of birth of ewes was studied by the one-way analysis model of variance ANOVA. A certain superiority was observed in the examined traits of the animals born as twins and triplets, but no significant differences were found between the individual groups.

Key words: *birth type, FABP3 gene, litter size, sheep.*

INTRODUCTION

FABPs are members of a family of conserved intracellular lipid-binding proteins with low molecular weight and high binding capacity for long-chain fatty acids, and 9 tissue-specific cytoplasmic FABPs (FABP1-FABP9) have been identified so far, and they are differentially expressed in different tissues (Chmurzynska, 2006). These small intracellular proteins having a molecular size of 14 to 16 kDa with 126 to 134 amino acids (Lang et al., 2017) and they are found in all animal species (Cho et al., 2011; Wang et al., 2015; Wang et al., 2016; El-Mansy et al., 2019; Al-Janabi, 2019; Ye et al., 2022). FABPs regulate intracellular levels of fatty acids and thereby control various cellular processes and lipid metabolism, cell growth and proliferation (Kulig et al., 2013).

The FABP3 protein is involved in the transport and exchange of fatty acids from the cell membrane to intracellular sites for fatty acid utilization (Veerkamp et al., 1995) and is predominantly expressed in tissues with a high demand for fatty acids such as cardiac and skeletal muscles, mammary gland during of lactation, liver or adipose tissues (Calvo et al, 2004; Lanier and Corl, 2015). Among the FABPs, FABP3, also known as Heart FABP (H-FABP) because it is mainly expressed in cardiac muscle and was originally isolated there (Gerbens et al., 1999).

The fatty acid binding protein 3 (*FABP3*) gene is an important candidate-gene for both the quality of the meat and the properties of dairy products such as the production of cheese due to its possible effects on the content of dairy fat (Calvo et al, 2004). The genetic variants of *FABP3* have been reported to affect the content of intramuscular fat in both sheep and pigs, and in pigs is considered to be a candidate-gene for fat characteristics (Gerbens et al., 1999; Uemoto et al., 2007). In the sheep, the *FABP3* gene is mapped in the distal part of Chromosome 2 of *Ovis aries* genome. Sheep gene FABP3 and its chromosomal location were established in 2002 (Calvo et al., 2002) and as a result 13 SNPs, one CTC insertion/deletion and a variable polyA tract have been found. Two of the established SNPS, located in exon 2 and intron 13, respectively, have been studied for associations and found that heterozygous genotypes for the two SNPS are related to the milk fat content (Calvo et al., 2002; Oner et al., 2014).

Studies in pigs have shown that the *FABP3* gene is associated with carcass fat content, intramuscular fat and meat quality, and its main functions are to regulate fatty acid uptake and intracellular transport (Chmurzyńska, 2006; Hong et al., 2015). The *FABP3* gene can be used as a genetic marker for increased intramuscular fat content (Chen et al., 2014; Gondim et al., 2019). Adipose tissue, in turn, often affects reproductive traits, as it plays an endocrine role influencing the metabolism of sex hormones (Pinto, 2014).

The reproductive performance is an indicator of reproductive efficiency and degree of genetic progress in both crossbreeding and selection programs (Nuraddis et al., 2011). Selection using a gene marker complements traditional methods, allowing selection of animals with higher accuracy and at an earlier age. Identifying candidate genes responsible for phenotypic variation is challenging because they are usually controlled by many genes and influenced by environmental factors (Andersson, 2001; Al-Janabi, 2019).

Prolificacy is the main economically significant trait for sheep of all breeds, and according to some authors, the number of lambs born per lambing was more important than the profit generated from the realized lambs (Petrović, 2012). Notter et al. (2000) indicated that fertility also determined the biological performance of sheep.

Sheep from the Bulgarian Dairy Synthetic Population are the most numerous, commercial and adaptable breed in Bulgaria. The animals have the potential for high milk yield - from 150 to 200 l (for the milking period) and good fecundity - 150 lambs from 100 ewes. Regarding fertility, the goal of selection is to increase the number of ewes having mainly 2 lambs per birth (Stancheva et al., 2014). It is known that the improvement of economically important traits by traditionally known selection methods will take a long period of time. Knowledge of the genetic background of sheep under selection control is of paramount importance to increase animal productivity and production efficiency. Therefore, it is necessary to accumulate sufficient information about their genetic diversity and the relationship of established polymorphic variants with economically useful traits. This is extremely important for commercial breeds that are grown in very different production systems and have difficulty expressing their genetic potential for high productivity. In Bulgaria, studies to identify a genetic polymorphism of the FABP3 gene in sheep of different breeds are limited, and an association of the established polymorphism of FABP3 with the fertility of sheep was performed in only one of them (Dimitrova et al., 2021).

The aim of the study is to investigate the relationship of the FABP3 gene polymorphism with the litter size in ewes from the Bulgarian Dairy Synthetic Population.

MATERIALS AND METHODS

Subject of the study were ewes from the Bulgarian Dairy Synthetic Population, raised at the Agricultural Institute - Shumen (Figure 1). The flock was created according to a kind of modified scheme, as a genealogical structure was formed and built already at the stage of the applied crossing schemes (Stancheva, 2003; Stancheva et al., 2014; 2016; 2017). For more than 30 years, "interlinear breeding" has been carried out with rams of our own production, applying homogeneous selection combined with moderate inbreeding. The sheep were divided into 3 flocks and were raised in a semi-intensive system. Young animals were kept separately until they reached 18 months and entered the main flock. Their feed was our own production. The breeding process took place as standard - once a year, in the months of June - July. Sheep were artificially inseminated according to an individual plan at random at 18 months after the formation of the flocks. Lambing was usually performed from the second half of November and ends by the middle of January.

A total of 110 ewes of different ages were included in the study. Animals were selected by birth type (singles, twins, triplets), with records of the number of lambs born from a minimum of two consecutive litters. A total of 433 records of the number of lambs born from a mother ewe were analysed, and the average number of lambs from the studied sample was 3.94.

Figure 1. Ewes from the herd of Bulgarian Dairy Synthetic Population in the Agricultural Institute - Shumen

The experimental work on DNA analysis was carried out in the Laboratory of Genetics at the Faculty of Agronomy of the Forestry University, Sofia, Bulgaria. Peripheral blood samples were stored in tubes containing EDTA at -20°C until the DNA extraction process. DNA was extracted by manual commercial kit for DNA purification according to the instruction (QIAamp DNA Blood Mini Kit Qiagen).

The polymerase chain reaction amplifications were performed in total volumes of 10 µl, containing 4 µl DNA template, 0.2 µl sterile H_2O, 0.4 µl of each primer and 5 µl of 2 × (1.5 mM $MgCl_2$) MyTaq TM HS Red Mix 2x (Bioline, UK). The primer set used was suggested by Calvo et al. (2004):

F: 5′-GGTTTTGCTACCAGGCAGGT-3′ and

R: 5′-TTCCCTATTCCCCTTCAGGG-3’.

PCR amplifications were effectuated by thermal cycler QB-96 (Quanta Biotech) under the next conditions: primary denaturation at 94ºC for 2 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 30 s, elongation at 72ºC for 1 min, and final extension at 72ºC for 10 min.

All animals were genotyped using RFLP analysis. The amplification products of the *FABP3* gene fragment (exon 2 - 222 bp) were processed separately in 10 µl final volume, containing 6 µl PCR product, 2.5 µl dd H_2O, 10 U/µl restriction enzyme *BseDI* (Thermo, US) and 1µl enzyme buffer. The digestion reactions were fulfilled at 60˚C for 3 h in thermal block. The fragment sizes were identified using Ready-to-Use DNA Ladder, 50 bp (Thermo, US) on 2,5 % agarose (Bioline) gel and stained by RedGel Nucleic Acid Stain (Bioline, UK). The PCR products and restriction fragments were visualized under UV light.

The allelic and the genotypic frequencies of *FABP3* gene were estimated using simple gene counting method (Falconer and Mackay, 1996). The association of *FABP3* gene polymorphism with total litter size and litter size depending on the parity and type of birth of ewes was established using the one-way analysis model of variance ANOVA.

RESULTS AND DISCUSSIONS

A 222 bp fragment from exon 2 of *FABP3* locus of sheep was amplified using the PCR technique from each of 110 ewes. The obtained PCR products were cut with restriction enzyme *BseDI*. Two alleles - mutant *G* (with 143, 43 and 36 bp fragments) and wild *A* (with two fragments -186 and 36 bp) were detected (Table 1). In the studied Bulgarian Dairy Synthetic Population ewes from flock of the Agricultural Institute - Shumen were identified both possible alleles mutant *G* and wild *A* with frequency 0.86 and 0.14, respectively. Two different genotypes were identified in SNP3 of fatty acid-binding protein 3 gene - homozygous genotype *GG* with frequency 0.73 and heterozygous genotype *AG* with frequency 0.27. Observed heterozygosity (H_o) was 0.272 and expected heterozygosity (H_e) was 0.240. This flock was found to be in Hardy-Weinberg equilibrium.

In our previous study with 30 ewes from the same herd, the allelic frequency was similar as two alleles and two genotypes were found with frequencies of 0.67 for *GG* and 0.33 for *AG* (Dimitrova et al., 2022). Earlier, Dimitrova et al. (2021) also found the presence of two alleles (*A* and *G*) and two genotypes (*AG* with a frequency of 0.30 and *GG* with a frequency of 0.70) in BDSP sheep reared in Institute of Animal Science - Kostinbrod. In another study, this region of the *FABP3* gene was examined in Bulgarian sheep from three merino and two local breeds (Dimitrova et al., 2020). Two alleles were found with the frequency of allele *G* ranging from 0.77 to 0.87, and allele *A* - from 0.13 to 0.23. All three possible genotypes were identified in all five breeds with the frequency of *GG* genotype - from 0.57 to 0.80, of AG genotype - from 0.13 to 0.40 and of AA genotype - from 0.03 to 0.07. Oner et al. (2014) also identified all three possible genotypes of the FABP3 gene in Kıvırcık sheep bred in three different provinces of Turkey. The authors found genetic diversity in the same region of the *FABP3* gene - SNP3, with the frequency of allele *A* being 0.42 and allele *G* - 0.58. In contrast to the present study, homozygous genotype AA was observed in this Turkish breed with a frequency of 0.30, while the other two genotypes - *GG* (0.46) and *AG* (0.24) had a lower frequency than found in our study.

Our obtained mean litter size value for the Bulgarian Dairy Synthetic Population ewes from the studied flock was 1.59 with trait-specific phenotypic variation (37.73%) (Table 2). In a similar study, Dimitrova et al. (2021) found a lower value for litter size (1.26) for ewes from the same population bred at Institute of Animal Science - Kostinbrod. On another scale, the size of the litter varies from 1.46 pcs. lambs born in the 2nd lambing to 1.66 number of lambs born in the 5th lambing, and according to the type of birth of the sheep, there is a certain superiority of the animals born as twins and triplets. In the analysis of variance, no significant differences were found between the individual groups.

The results for the litter size of the established genotypes are presented in Table 3. Carriers of the homozygous genotype *GG* were 72.72% of the animals studied. Their litter size was 0.45 higher than that of ewes with the heterozygous *AG* genotype, but the differences were not statistically significant. In contrast to us, Dimitrova et al. (2021) found a proven higher litter size in sheep of the same population with a heterozygous genotype of the *FABP3* gene, in the flock of Institute of Animal Science - Kostinbrod. The results for the litter size by successive weaning and the type of birth of the ewes for the two genotypes did not outline a clear trend of superiority of the animals with the homozygous genotype *GG*. The established differences between the individual groups again have no statistical significance.

CONCLUSIONS

The results obtained in this study indicate that fatty acid binding protein 3 in sheep is a polymorphic gene. Genetic diversity was found in SNP3 (the exon 2) of the *FABP3* gene in the 110 animals studied of Bulgarian Dairy Synthetic Population - two alleles (wild *A* and mutant *G*) and two genotypes (homozygous *GG* and heterozygous *AG*). The *G* allele and the homozygous *GG* genotype show a higher frequency. The mean litter size for ewes from the study flock was 1.59, which ranged from 1.46 at 2nd lambing to 1.66 at 5th lambing. According to the type of birth of the sheep, there is a tendency for superiority of those born as twins and triplets. Carriers of the homozygous genotype *GG* show a tendency for a higher litter size compared to ewes with the heterozygous genotype *AG*.

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Table 1. Allele and genotype frequencies of SNP3 of *FABP3* gene

Locus	n	Allele frequency		Genotype frequency			Heterozygosity		Fis	χ^2	p
		G	*A*	*GG*	*AG*	*AA*	*Ho*	*He*			
FABP3	110	0.86	0.14	0.73	0.27	0.00	0.272	0.240	-0.133	3.62	0.05

Table 2. Overall mean for litter size

Variable	n	Average	C.V. %	P-value
Total litter size	433	1.59	37.73	
Liter size by parity				
1-st lambing	110	1.61	38.71	0.268
2-nd lambing	110	1.46	28.77	
3-rd lambing	95	1.65	42.06	
4-th lambing	70	1.63	49.77	
5-th lambing	32	1.66	29.74	
6-th lambing	16	1.56	26.25	
Liter size by type of sheep birth				
Singles	171	1.51	33.36	0.162
Twins	215	1.63	41.23	
Triplets	47	1.64	36.63	

Note. *denotes a statistically significant difference.

Table 3. Association of FABP3 gene polymorphism with litter size

Variable	Genotype *AG*			Genotype *GG*			P-value
	n	Average	C.V. %	n	Average	C.V. %	
Total liter size	119	1.55	33.39	314	1.60	39.44	0.505
Liter size by parity							
1-st lambing	30	1.60	31.72	80	1.61	41.76	0.926
2-nd lambing	30	1.37	24.02	80	1.50	30.38	0.247
3-rd lambing	26	1.62	48.61	69	1.67	40.20	0.733
4-th lambing	18	1.61	36.93	52	1.63	55.02	0.904
5-th lambing	10	1.60	26.67	22	1.68	32.25	0.701
6-th lambing	5	1.80	20.00	11	1.45	27.27	0.223
Liter size by type of sheep birth							
Singles	54	1.52	32.98	117	1.51	33.82	0.952
Twins	44	1.61	38.21	171	1.63	42.23	0.869
Triplets	21	1.52	26.19	26	1.77	42.46	0.166

Note. *denotes a statistically significant difference.

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THE CHEMICAL COMPOSITION AND NUTRITIONAL VALUE OF THE PLANT MASS OF THE NEW HYBRID OF SORGHUM - SUDAN GRASS 'SAȘM-4' GROWN UNDER THE CONDITIONS OF MOLDOVA

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Abstract

To establish a fodder base under the conditions of climate change, it is necessary to use new plants species, cultivars and hybrids, which are more resistant to droughts and high temperatures. One of these plants is the new sorghum - Sudan grass hybrid 'SAȘM 4'. The goal of our research was to determine the dynamics of the chemical composition and nutritional value of these plants harvested in different developmental periods: stem elongation, tasselling, milk-wax and wax stage of grains, as well as the capacity of being processed into silage. It was determined that the dry matter content in the harvested green mass varied depending on the harvest time from 130.0 g/kg in the stem elongation period to 340.1 g/kg in the wax stage of grains, its chemical composition and nutritional value were: 6.53-18.40% crude protein, 2.05-3.86% crude fats, 28.62-37.61% crude cellulose, 38.90-54.84% nitrogen free extract, 7.71-10.56 % sugars, 1.43-11.94% starch, 5.25-10.22 % ash, 0.20-0.30% calcium, 0.13-0.26% phosphorus, 31.85-53.00 mg/kg carotene, 0.12-0.26 nutritive units/kg natural fodder and 1.29-2.96 MJ/kg natural fodder metabolizable energy. The fermentation quality and fodder value of silage prepared from the sorghum- Sudan grass hybrid 'SAȘM-4' were: pH = 4.06, 19.8 g/kg lactic acid, 6.9 g/kg acetic acid, butyric acid was not detected, 334.8 g/kg DM, 7.05% crude protein, 2.55% crude fats, 34.05% crude cellulose, 51.12% nitrogen free extract, 1.03% soluble sugars, 9.96% starch, 5.22% ash, 0.22% calcium, 0.15-0.27% phosphorus, 23.75 mg/kg carotene, 0.26 nutritive units/kg silage and 2.99 MJ/kg silage metabolizable energy.

Key words*: chemical composition, green mass, nutritional value, silage, sorghum - Sudan grass hybrid 'SAȘM-4'*

INTRODUCTION

Milk and meat are some of the most valued products for human beings produced by ruminant livestock. Adequate animal nutrition is one of the most important factors which determine not only the quantity but the quality of the milk and meat produced.

The diversification of fodder sources for the animal husbandry sector of the Republic of Moldova is a necessity dictated by several factors, but primarily by climate change, which imposes the need to use new, lesser-known fodder plants that are more resistant to high temperatures and insufficient rainfall.

The plants with C_4 carbon fixation have a particular leaf structure, the so-called Kranz anatomy, with 2 types of assimilatory cells: a layer of mesophyll cells surrounding a inner layer of bundle sheath cells enclosing like a ring the vascular bundle, the process of photosynthesis takes place inside the cells and is faster than in C_3 plants under conditions of intense light and high temperatures because CO_2 is supplied directly to ribulose bisphosphate carboxylase (RUBISCO), not allowing the assimilation of O_2 and photorespiration, they have a better water use efficiency because phosphoenolpyruvate carboxylase (PEP Carboxylase) quickly transports CO_2 and there is no need for the stomata to be open for too long (thus reducing water loss through transpiration) for the same amount of CO_2 gained for photosynthesis (Petcu, 2008).

Among C_4 plants, the genus *Sorghum* Moench is of particular interest. It belongs to the tribe *Andropogoneae,* subfamily *Panicoideae,* family *Poaceae*, which includes 31 species, native to Europe, Asia, North and South

America, as well as Australia. *Sorghum* species have recently gained popularity due to their numerous advantages, such as heat and drought tolerance, resistance to specific diseases and pests, being able to exploit the salty soils where the cultivation of cereals is more difficult. The adaptive nature of *Sorghum* species as C_4 plants and the better water use efficiency, their potential to produce higher yields of grains or green forage and their diverse uses make them a valuable "tool" and one of the best choices for forage growers and dairy farmers demanding high quality feed stocks, also for food and other industrial uses, production of cellulose or renewable energy (Moraru, 2008; Voicu et al., 2013; Herrmann et al., 2016; Roman et al., 2016; Wannasek et al., 2017; Zhang et al., 2021). *Sorghum* grains can be used to produce gluten-free foods, can be given to sheep, pigs and even poultry, but are usually ground for cattle (Marin et al., 2016).

In our region, in the 17th century, sorghum was introduced to make brooms - *Sorghum technicum* (Körn.) Trab., and during the last century, other species were also introduced: for grains - *Sorghum bicolor*, for fodder - *Sorghum sudanense*, and for the food industry *Sorghum bicolor* var. *saccharatum* and *Sorghum bicolor* var. *oryzoidum,* as well as *Sorghum* × *almum* - also for the production of fodder (Moraru, 2008; Țîței et al., 2019).

Sorghum bicolor (sorghum) has been widely used for the production of forage and silage to feed animals, besides being used as a grain and energy crop. Its leaves are broad, have high palatability and provide green fodder over a longer period, but *Sorghum bicolor* is not a multi-tillering and multicut. On the other hand, *Sorghum sudanense* (Sudan grass) is a multicut and multi-tillering fodder plant but its leaves are narrow, having low palatability. Therefore, it became needed to converge the favorable characters of sorghum and Sudan grass to develop a multicut and multi-tillering plant producing palatable green fodder. The sorghum-sudangrass hybrids obtained when crossing *Sorghum bicolor* (L.) Moench and *Sorghum sudanense* (Piper) Stapf, have been well accepted by cattle farmers because they have flexible planting times and high production potential, and are an option for intensifying animal production, especially at times of feed shortage. The importance of sorghum-Sudan grass hybrids as annual forage in the composition of diversified feeding systems has combined with the increasing demand for forages of greater nutritional value. Hybrids between sorghum and Sudan grass take the positive parts of both species: from sorghum - the capacity to grow taller and a higher sugar content, from Sudan grass - the ability to regenerate quickly after mowing, which gives the possibility to cut the plants up to three times per year. Sorghum - Sudan grass hybrids offer a solution to producing forage when other fodder crops are not available and emergency occurs. In comparison with maize, sorghum - Sudan grass hybrids can generate dry matter in similar quantities for silage, has equivalent yield potential and has greater water use efficiency and drought resistance (Getachew et al., 2016).

In the Republic of Moldova, for several years, research has been carried out in order to obtain hybrids between sorghum and Sudan grass (Moraru, 1989; 2008; Chisnicean, 1995). One of these hybrids, recently obtained at the Institute of Genetics, Physiology and Plant Protection, is the SAȘM-4 hybrid (BOPI 11/2022), which, as claimed the authors, can be used to obtain green mass, produce silage, haylage and hay, but in-depth research in this regard has not been carried out yet. The main goal of the research carried out by us was to determine the chemical composition and the nutritional value of the plant mass of this hybrid, harvested in various stages of development, as well as its capacity to be ensiled.

MATERIALS AND METHODS

The sorghum - Sudan grass hybrid 'SAȘM-4' which was cultivated in the experimental plot of the Plant Resources Laboratory of the National Botanical Garden (Institute), N 46°58′25.7″ latitude and E 28°52′57.8″ longitude, served as subject of the research.

The plant samples were collected in 4 periods: stem elongation, tasselling, milk-wax stage of grains and wax stage of grains. The harvested plants were chopped into 1.5-2.0 cm small pieces, with a laboratory forage chopper, the dry matter content was detected by drying samples up to constant weight at 105°C. The chopped mass samples were dehydrated in an oven with forced ventilation at a temperature of 60°C; at the end of the fixation, the biological material was finely ground in a laboratory ball mill. The preparation of silage and the evaluation of its quality were carried in accordance with the methodological indications and the requirements of the **Moldavian standard SM 108. The chopped green mass was compressed in well sealed glass containers, stored at ambient temperature (18-20°C) for 45 days, to allow complete fermentation to occur. Following the 45-day fermentation period, each glass container was opened and the content was visually examined, the colour and the aroma were recorded. The pH of the samples of silage was measured immediately after removal from the containers. At the same time, samples were taken to determine the content of organic acids (lactic, acetic and butyric) in free and fixed state. The evaluation of chemical composition: crude protein (CP), crude fat (EE), crude cellulose (CF), nitrogen-free extract (NFE), soluble sugars (SS), starch, ash, calcium (Ca), phosphorus (P), carotene, content of organic acids in silage were carried out in the Laboratory of Nutrition and Forage Technology of the Scientific-Practical Institute of Biotechnology in Animal Husbandry and Veterinary Medicine, in accordance with the methodological indications. The nutritive units and metabolizable energy were calculated according to standard procedures (Kalashnikov et al., 2003).

RESULTS AND DISCUSSIONS

The adequacy of nutrients supplied by feed is an essential factor for animal performance. Feed should contain a satisfactory concentration of proteins and nonfibrous carbohydrates (starch and soluble sugars), the latter being the most important source of energy produced in the rumen. This concentration stimulates the growth of bacteria and increases the production of microbial protein and volatile fatty acids necessary to induce an optimal productivity of milk or meat in livestock. To provide animals with high quality forage, practical methods are needed to estimate nutritive value to optimize harvest timing.

Table 1. The chemical composition and nutritional value of the green mass of depending on the harvest time of sorghum - Sudan grass hybrid 'SAȘM-4'

Indices	Sorghum - Sudan grass hybrid 'SAȘM-4'				Corn hybrid 'Porumbeni 374' wax stage of grains
	stem elongation stage	tasseling stage	milk-wax stage of grains	wax stage of grains	
Dry matter, g/kg GM	130.0	226.3	321.4	340.1	320.2
Crude protein, % DM	18.40	8.47	6.16	6.53	7.26
Crude fats, % DM	3.86	2.75	2.05	2.19	2.83
Crude cellulose, % DM	28.62	37.61	32.83	30.94	18.40
Nitrogen free extract, % DM	38.90	45.19	53.71	54.84	67.92
Soluble sugars, % DM	7.91	10.56	9.43	7.71	7.55
Starch, % DM	1.43	1.50	8.23	11.94	22.79
Ash, % DM	10.22	5.99	5.25	5.49	3.59
Nutritive units/ kg GM	0.12	0.23	0.24	0.26	0.32
Metabolizable energy, MJ/kg GM	1.29	2.33	2.82	2.96	3.33
Calcium, % DM	0.31	0.20	0.24	0.30	0.24
Phosphorus, % DM	0.26	0.13	0.14	0.14	0.22
Carotene mg/ kg	53.00	32.96	35.00	31.85	14.30

The data on the chemical composition of the green mass of the sorghum x Sudan grass 'SAȘM-4' plants (Table 1) harvested in the stem elongation stage, when the plants were about 1.25 m tall, show that it is characterized by a low content of dry matter, but very high - of crude protein (18.40%), crude fat (3.86%), carotene (53.00 mg/kg), ash (10.22%), calcium (0.31%) and phosphorous (0.26%). The energy load of the plant in this stage of development is only 0.12 nutritive units/kg or 1.29 MJ/kg, but relatively to the dry matter content, the nutritional value is very high: 0.92 nutritive units/kg and 9.53 MJ/kg metabolizable energy.

In a more advanced stage of development - the tasselling stage, the plants of this hybrid had a lower moisture content of 77.37%, the content of crude protein and crude fat decreased sharply, and the amount of crude cellulose and Nitrogen free extract increased essentially as compared with the previous plant development stage. The sugar content increased and reached 10.56%, while the starch content varied insignificantly. The content of mineral substances, phosphorus and calcium decreased sharply, and the amount of carotene decreased even more significantly. The energy load of the natural feed harvested in this development stage increased up to 0.23 nutritive units/kg and 2.33 MJ/kg metabolizable energy.

The determination of the content of dry matter and its chemical composition in the fodder harvested in the milk-wax stage of grains demonstrated an essential increase in the dry matter content and a decrease in the content of crude protein, crude fat and crude cellulose. A significant increase in nitrogen free extract and starch content was found in comparison with the previous harvest period. The energy load of the natural feed harvested in this development stage reached 0.24 nutritive units/kg and 2.82 MJ/kg metabolizable energy.

The sorghum x Sudan grass plants harvested in the wax stage of grains are characterized by a higher content of dry matter, and especially of starch and calcium, a decrease in the content of crude cellulose and soluble sugars as compared with the previous harvest stage. The sorghum x Sudan grass natural forage harvested in the wax stage of grains has a higher content of dry matter than maize. The content of nutrients differs essentially; the sorghum x Sudan grass fodder has a higher content of crude cellulose, ash, phosphorus and carotene and - lower of nitrogen free extract and starch, which has a negative impact on the energy input of the feed.

Several literature sources describe the productivity and nutritional value of sorghum - Sudan grass hybrids. According to Chisnicean (1995), the best sorghum - Sudan grass lines produce more than 19-20 t/ha dry matter with 13-15 % protein. Burlacu et al. (2002) revealed that *Sorghum bicolor x sudanense* green forage harvested in the tasseling period contained 200 g/kg DM, 16.3% CP, 4.2% EE, 26.6% CF, 41.8% NFE, 11.1% ash and 18.3 MJ/kg GE, in flowering period - 300 g/kg DM, 9.4% CP, 3.1% EE, 29.7% CF, 47.8% NFE, 10.0% ash and 17.8 MJ/kg GE, but in milk stage of grains - 332 g/kg DM, 4.8% CP, 2.4% EE, 36.5% CF, 48.2% NFE, 8.1% ash and 17.9 MJ/kg GE. Pospišil et al. (2009) remarked that forage sorghum, hybrid Grazer N (*Sorghum bicolor* x *S. sudanense*), harvested in the period when plants were 100 cm tall, contained 13.5-14.6% CP, 9.4-9.8% DP, 3.3-3.4% EE, 23.3-27.2% CF, 57.6-63.4% NDF, 28.9-33.7% ADF; the forage harvested when the plants were 150 cm tall contained 9.6-12.8% CP, 6.6-8.2% DP, 2.0-2.8% EE, 25.8-31.2% CF, 63.6-69.3% NDF, 30.2-38.5% ADF, but forage harvested in the tasselling stage - 6.4-9.1% CP, 4.3-6.5% DP, 1.9-2.1% EE, 29.5-32.2% CF, 63.7-65.9% NDF, 35.2-38.9% ADF, respectively. Uzun et al. (2009) mentioned that total fresh herbage yields of eight sorghum x Sudan grass hybrid cultivars grow under the ecological conditions of Samsun, Turkey, varied from 50.04 t/ha to 97.41 t/ha, the nutritional and chemical properties of first-harvest dry matter were 6.82-9.03% CP, 6.12-7.76% ash, 66.04-74.89% NDF, 40.24-48.32% ADF, RFV=63.78-81.53, 0.40-0.67% Ca, 0.21-0.30% P, 1.37-1.51% K, 0.14-0.20% Mg. Glamoclija et al. (2011) found that the chemical compositions of dry biomass samples of sorghum - Sudan grass harvested in stem elongation period was 11.13-13.48% CP, 5.56-6.74% DP, 2.13-2.24% EE, 29.07-30.88% CF, 43.01-45.24% NFE, 10.58-11.32% ash, but the sorghum - Sudan grass plants harvested in tasseling period contained 10.04-11.26% CP, 5.02-5.64% DP, 2.14-2.32% EE, 30.65-32.67% CF, 43.48-45.33% NFE, 10.36-10.48% ash. Kerckhoffs et al. (2011) revealed that the dry matter content and biomass composition of sorghum cultivars were 250-350 g/kg DM, 4.2-6.4% CP, 1.2 % EE, 6.6-13.2 % sugars, 1.1-1.3% starch, 28.7-31.5% cellulose, 23.0-24.0% hemicellulose, 31.2-33.2% CF, 57.1-60.6% NDF, 34.0-36.7% ADF, 5.2-5.3% lignin, 4.8-6.2% ash. Mahmood et al. (2013) reported that the dry matter content and the chemical composition of green mass of the cultivar 'Bovital' of sorghum - Sudan grass was 205-265 g/kg DM, 8.0-11.1% CP, 51.1-59.5% NDF, 4.4-5.2%

ADL, 7.2-11.5% sugar and 8.3-9.8% ash, but the sorghum cultivar 'Goliath' contained 165-243 g/kg DM, 7.5-10.4% CP, 53.1-61.5% NDF, 4.1-5.4% ADL, 6.9-15.5% sugar and 8.4-9.4% ash, respectively. Ferreira et al. (2015) reported that the dry matter content and nutritional quality of sorghum - Sudan grass whole plants during the growth stages from 51 to 74 days after seeding changed: 8.14-18.80% DM, 8.67-14.16% CP, 63.6-70.7% NDF, 35.3-44.4% ADF, 2.33-4.68% lignin and 540-613g/kg IVDDM. Gelley et al. (2016) found that the concentrations of nutrients in sorghum - Sudan grass harvested at 4 weeks post monthly initiation was 9.30-12.21% CP, 60.47-66.52% NDF, 39.64-44.29% ADF and 520.9-597.7 g/kg NDFD, but in the plants harvested at 8 weeks post monthly initiation, there was 7.00-9.78% CP, 69.67-70.18% NDF, 45.39-46.45% ADF and 475.3-521.5 g/kg NDFD, respectively. Temel et al. (2017) mentioned that the chemical composition, energy and nutritional value of the green mass of sorghum - Sudan grass hybrids were: 8.74-8.88% CP, 64.97-65.64% NDF, 37.60-37.95% ADF, 5.53-6.39% ADL, 59.33-59.60% DDM, 2.80-2.82 Mcal/kg DE, 2.30-2.31 Mcal/kg ME, RFV = 84.38-85.32, but a Sudan grass variety contained 6.66% CP, 65.26% NDF, 40.44% ADF, 7.07% ADL, 57.39% DDM, 2.72 Mcal/kg DE, 2.23 Mcal/kg ME, RFV = 81.82. Ferreira et al. (2018) revealed that sorghum - Sudan grass hybrids obtained by conventional breeding were characterized by 7.97-8.27% DM, 13.4-14.0% CP, 61.6-64.8% NDF, 38.8-41.9%ADF, 3.52-4.12% lignin, 56.74-58.10% TDN, but brown-midrib sorghum - Sudan grass hybrids – 7.10-7.69% DM, 15.2-18.2% CP, 54.0-59.6% NDF, 33.3-38.1%ADF, 3.39-3.61% lignin, 58.94-61.28% TDN. Nohong & Islamiyati (2018) mentioned that hybrid Sudan grass reached 196.39 cm in height and produced 13.74 t/ha dry matter forage with 6.97% CP, 38.72% ADF, 69.46% NDF, 5.56% ADL, 56.08% IVDMD and 56.27% IVOMD. Machicek et al (2019), revealed that the dry matter yield and herbage quality of a sorghum - Sudan grass cultivar, harvested in 30 days after emergence, was 1.06-1.83t/ha DM with 10.6-11.0% CP, 34.7-35.8% ADF, 58.3-62.1% NDF, 62.6-63.5% TDN, RFV= 92.8-97.3; the plants harvested in 45 days contained 2.65-4.59 t/ha DM, 5.8-9.9% CP, 38.9% ADF, 62.1-63.9% NDF, 59.0% TDN, RFV= 85.3-87.8, but in 90 days - 6.29-9.87 t/ha DM, 4.3-5.1% CP, 38.0-39.3% ADF, 59.9-64.5% NDF, 58.6-59.8% TDN, RFV= 85.5-90.8, respectively. Paradhipta et al. (2019) reported that the dry matter content, the chemical composition and *in vitro* digestibility of sorghum - Sudan grass forages were: 228-233 g/kg DM, 11.2-11.8% CP, 2.80-3.41% EE, 8.64-9.13% ash, 37.3-37.9% ADF, 67.3-67.5% NDF, 57.4-58.4% IVDMD and 52.9-53.8% IVNDFD. Rihacek et al. (2020), mentioned that studied Sudan grass hybrids were characterized by 9.7-11.2% CP, 50.1-55.6% NDF, 29.1-35.0% ADF, 45.0-55.3% IVDDM and 39.3-50.0% IVDOM, but sorghum grains varieties 11.1-11.3% CP, 48.0-50.5% NDF, 27.3-29.8% ADF, 52.8-55.0% IVDDM and 47.2-50.0% IVDOM.

The proportion of conserved forages significantly increased in relation to the total yearly feed production, and the feed quality has markedly improved during the last 50 years. During times of plentiful growth, fodders can be stored as silage or hay. Currently, silage is the most common source of preserved feed for ruminant animals. Silage, when formed properly, provides the same or even higher value as ensiled fodder. Because of its relished consumption, good quality silage can increase animal health and. Silage plays an important role in the nutrition, wellbeing and productivity of animals. It can help solving some problems in the livestock sector by providing a balanced diet for animals with an appropriate amount of protein and fibre. As for the organoleptic properties, the silage prepared from sorghum - Sudan grass hybrid 'SAȘM-4' had yellowish-green colour with pleasant smell of pickled vegetables; the texture of the plants stored as silage was preserved well, without mold and mucus. The fermentation quality and fodder values of silage prepared from the sorghum - Sudan grass hybrid 'SAȘM-4' and the maize hybrid 'Porumbeni 374' are shown in Table 2. It has been determined that pH values 3.92-4.06 and the amounts of organic acids in the prepared silage reached 26.7-36.7 g/kg, most organic acids were in fixed form, butyric acid not was detected and lactic acids constituted 74-76%. The sorghum - Sudan grass silage was characterized by low content of organic acids, in comparison with the maize silage. The dry matter content in the prepared silages varied from 319.5 g/kg in maize silage to 334.8 g/kg in sorghum - Sudan grass silage, its nutrient content was: 7.05-7.28% CP, 2.55-3.94% EE, 19.02-34.05% CF, 51.12-66.22% NFE, 0.91-1.03% soluble sugars, 9.96-24.54% starch, 3.55-5.22% ash, 0.22-0.27% Ca, 0.15-0.27% P, 23.75-28.02 mg/kg carotene. In comparison with the initial mass, in the prepared silages the level of soluble sugars decreased substantially, but crude protein, crude fats, crude cellulose, nitrogen free extract and ash did not change essentially. The dry matter of sorghum - Sudan grass silage contained a low amount of crude fats, starch and a high amount of crude cellulose as compared with the traditional silage crop - maize. It has been calculated that 100 kg of silage prepared from sorghum - Sudan grass hybrid contained 26 nutritive units, 2.36 kg crude protein and 299 MJ metabolizable energy, but maize silage - 30 nutritive units, 2.33 kg crude protein and 325 MJ metabolizable energy.

Table 2. The fermentation quality, chemical composition and nutritional value of the of the investigated silage

Indices	Sorghum - Sudan grass hybrid 'SAȘM-4' wax stage of grains	Corn hybrid Porumbeni 374 wax stage of grains
pH index	4.06	3.92
Total organic acids, g/kg DM	26.7	36.7
Free acetic acid, g/kg DM	3.3	4.3
Free butyric acid, g/kg DM	0	0
Free lactic acid, g/kg DM	7.9	12.9
Fixed acetic acid, g/kg DM	3.6	4.6
Fixed butyric acid, g/kg DM	0	0
Fixed lactic acid, g/kg DM	11.9	14.9
Total acetic acid, g/kg DM	6.9	8.9
Total butyric acid, g/kg DM	0	0
Total lactic acid, g/kg DM	19.8	27.8
Acetic acid, % total acids	25.84	24.25
Butyric acid, % total acids	0	0
Lactic acid, % total acids	74.16	75.75
Dry matter, g/kg silage	334.8	319.5
Crude protein, % DM	7.05	7.28
Crude fats, % DM	2.55	3.94
Crude cellulose, % DM	34.05	19.02
Nitrogen free extract, % DM	51.12	66.22
Soluble sugars, % DM	1.03	0.91
Starch, % DM	9.96	24.54
Ash, % DM	5.22	3.55
Nutritive units/ kg silage	0.26	0.32
Metabolizable energy, MJ/kg silage	2.99	3.25
Calcium, % DM	0.22	0.27
Phosphorus, % DM	0.15	0.27
Carotene mg/ kg	23.75	28.02

Some authors mentioned various findings about the quality of silage from *Sorghum* species. Voicu et al. (2013) reported that the silage prepared from the sorghum cultivars F436 and F465, harvested in the milk-dough stage contained 6.39-6.74% CP, 1.23-1.38% EE, 36.8-39.3% CF, 45.1-48.4% NFE, 7.03-7.58% ash, 0.34-0.39% Ca, 0.14-0.21% P, 55.50-56.90% DDM, but maize silage - 6.57-6.63% CP, 2.91-3.16% EE, 17.26-17.62% CF, 5.89-6.70% ash, 0.23-0.26% Ca, 0.13-0.22% P, 66.35-68.18% DDM. Herrmann et al. (2016) mentioned that the silage from sorghum - Sudan grass hybrid was characterized by 245 g/kg DM, pH = 3.8, 6.7% lactic acid, 1.5% acetic acid, 8.9% CP, 1.8% EE, 52.2% NFE, 58.0% NDF, 36.6% ADF, 5.5% ADL and 5.7% ash, but forage sorghum silage 243 g/kg DM, pH 3.7, 6.7% lactic acid, 1.5% acetic acid,

8.0% CP, 1.5% EE, 52.3% NFE, 60% NDF, 38.6% ADF, 5.7% ADL and 5.7% ash. Oliveira et al. (2018) found that sorghum - Sudan grass silages had pH = 3.7-3.9 and contained 262.9-289.5 g/kg DM, 3.58-4.33% lactic acid, 0.75-0.1.14% acetic acid, 0.16-0.26% butyric acid, 4.72-5.14% ash, 2.08-3.21% EE, 43.39-58.73% NDF, 24.80-43.01% ADF, 32.40-43.39% NFC, 2.10-4.09% lignin, 54.19-74.88% TDN, 53.2-78.5% IVDMD. Paradhipta et al. (2019) determined that sorghum - Sudan grass silages had 172-186 g/kg DM, pH = 4.25-4.32, 1.89-2.08% lactic acid, 0.68-0.69% acetic acid, 0.81-0.89% butyric acid, 11.5-12.5% CP, 2.50-2.66% EE, 9.08-9.14% ash, 39.5-40.4% ADF, 68.3-68.6% NDF, 49.3-54.4% IVDMD and 56.4-56.8% IVNDFD. Ozkan (2022) revealed that pure silage from sorghum - Sudan grass harvested during at the mid-dough stage was characterized by 33.04% DM, pH = 3.99, 7.62% CP, 55.46% NDF, 37.75% ADF, 8.18% ash, 61.05% DDM, 9.34 MJ/kg ME and RFV = 107.42, but the silages made from different mixtures of sorghum - Sudan grass and sunn hemp contained 29.07-31.98% DM, pH = 4.07-4.32, 9.37-15.12% CP, 56.60-60.17% NDF, 37.12-42.56% ADF, 7.22-8.03% ash, 55.75-59.99% DDM, 8.32-9.13 MJ/kg ME and RFV = 86.20-98.60. Ramzan et al. (2022) reported that the dry matter content, the chemical composition and the nutritive value of silages from sorghum - Sudan grass were characterized by the following indices 232.5 g/kg DM, pH = 4.38, 7.89% CP, 7.51% ash, 61.33% NDF, 33.62% ADF, 4.22% lignin, 29.40% CEL, 27.72% HC, 63.02% DDM, 2.71 Mcal/kg DE, 2.33 Mcal/kg ME and RFV = 96.

CONCLUSIONS

The results of the conducted research allow us to conclude that the hybrid sorghum x Sudan grass SAȘM 4 has adapted to the arid climatic conditions of the Republic of Moldova, it is characterized by an optimal content of crude protein, in certain stages of development, a constantly increasing amount of sugar and starch and relatively high carotene content.

The downside of this crop is the comparatively high crude cellulose content.

Thus, in order to reduce the negative influence of droughts on the formation of the fodder base, widening the spectrum of fodder crops used in the diets of farm animals, we recommend the use of the new sorghum x Sudan grass hybrid 'SAȘM-4' as green as well as preserved fodder.

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QUANTITATIVE RESEARCH FOR CONSUMER PERCEPTIONS ON VEGETAL PROTEIN-RICH, NUTRITIONALLY BALANCED PRODUCTS

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Abstract

The modern lifestyle, with life being so busy with jobs, kids, and other activities, brings changes in eating habits, which has led to a significant reduction in daily number of traditional meals. Therefore, the market for protein-rich products, originally developed to increase the muscle mass of athletes, is growing supporting those who choose to replace traditional main meals as well as welcoming vegan and vegetarian consumers, whose number is constantly increasing. To meet the current demands of consumers, regarding the increase in the availability on the local market of healthy, minimally processed, protein-enhanced and last but not least nutritionally balanced products, which can be consumed by as many categories of consumers as possible, a study was carried out to establish consumer preferences for salty snack products with a high vegetable protein content. It involved the development of a questionnaire followed by its distribution in the online environment. The results obtained regarding the consumer preferences, allowed us to further establish the main characteristics and attributes of the desired product.

Key words*: consumer preferences, minimal processing, plant protein.*

INTRODUCTION

The eating habits of people have influences on both human health and the future of the planet, in terms of sustainability.

The high consumption of animal products has significant negative effects on the human health and the environment and is recommended to be reduced, thus encouraging the consumption of fresh and ecological vegetables and fruits, minimal processed (Van Loo et al., 2020). Furthermore, consumers are increasingly demanding food that is more sustainable and nutritious in order to improve or maintain their health and to contribute also to the environment protection.

Plant-based meat alternatives are being developed to meet consumer demands, thus the market has grown exponentially in recent years (Sha & Xiong, 2020; McClements & Grossmann, 2021).

Research and development of meat alternatives focuses on the production of sustainable products that recreate conventional meat with all its physical aspects (texture, appearance, taste, etc.) (Kyriakopoulou et al., 2021; Kurek et al., 2022).

Due to the negative effects related to the environment, and in order to align with the requirements of the European Union, circular economy must be applied, and in this sense the food industry sector from Romania managed to increase the use of by-products and valorize them into new food ingredients and products (Althumiri et al., 2021). Furthermore, several studies were performed in terms of by-products valorisation and application. For example, a study carried out in Portugal demonstrated that the by-products derived from the processing of green coffee (pulp, peel, mucilage, etc.) can be further used because of their high potential value for the development of active food packaging materials, and not discarded (Oliveira et al., 2021). Naik et al. (2023) investigated the valorisation of coconut mesocarp and their results showed that the processing of this by-product could lead to the obtaining of fibre rich products, minimising coconut processing waste. A group of researchers from the University of Castilla - La Mancha, Spain have shown that chia by-products obtained from the extraction of seed oil can be used to develop new biodegradable films for the food industry, specifically defatted chia flour can be used in the production of edible films with improved characteristics (Muñoz-Tebar et al., 2021). In Thailand, a Food and Nutrition Program at Chulalongkorn University analyzed unripe papaya by-products. A large volume of waste and by-products of unripe papaya is generated annually, so this study analysed the potential usage of these by-products as functional ingredients in pancakes, and the results were promising (Waralee et al., 2021).

In most areas where waste and by-products are obtained, the food industry is searching new ways to reuse or recover them. Grain waste from the beer industry represents about 85% of the total side products, thus catching the attention of many researchers for new ways to valorize them. He et al. (2023) stated that brewer's spent grain by-product is rich in proteins and fibers, also containing lipids, phenolic compounds and minerals. In Italy and Ireland these wastes were analyzed and the results obtained aroused the interest of specialists in the bread industry and beyond. Researchers at the University of Foggia, Italy, investigated by-products from the brewing industry and their use as functional ingredients in bread making. The results obtained showed significant increase in the phenolic content and insoluble and soluble dietary fibers of enriched bread. At the same time, a study conducted at the University College Cork in Ireland, showed that grain waste obtained from the brewing industry can serve as a raw material for the production of protein isolate and can be compared to pea and soy proteins in terms of essential amino acids content (Baiano et al., 2023; Jaeger et al., 2023).

In order to align with the current trends regarding the development of new plant-based products that respect the principles of a circular economy, the present paper analyzed the consumer preferences towards salty snack-type products, enriched in vegetable protein in order to assure nutritionally balanced products.

MATERIALS AND METHODS

Quantitative research allows the collection of information about a certain subject, using statistical methods. In the present study, the online survey (CAWI - Computer-Assisted Web Interviewing) was used (Quantitative research, 2023). The main advantages of using this method would be the quick receipt of answers from the participants (online), reduced analysis time, ease of correcting errors in a questionnaire and low cost. The method also has disadvantages, one being the reduction of the size of the population that can be surveyed due to the lack of access to the internet connection, the main participants being young people (Online Survey, 2023).

The study was carried out in 2022, between November and December and consisted of interviewing a number of 205 people. The interview was carried out by distributing a questionnaire designed on the basis of scientific literature and the requirements of the established objectives (Kotler, 1972; Honkanen, 2006). The questionnaire includes a number of 21 questions divided into two sections: 13 questions related to consumption behavior, 8 questions for creating the demographic profile and a third section consisting of a multidimensional scale for measuring consumer preferences for salty, vegetable snack-type products, with a high protein content and nutritionally balanced. The questions used in designing the questionnaire are closed dichotomous questions, questions with choice answers and open questions. Dichotomous closed questions are those to which only two answers can be given "yes" or "no", "man" or "woman". The questions with optional answers called semi-open are those that have a limited number of answers that are specified, and open questions are those to which the respondents can answer using their own words (ASE – Online library, 2021). In order to establish the consumer's perception towards snack-type, salty, high-protein and nutritionally balanced vegetable products, a set of 18 statements with a five-point Likert-type metric scale was used (total disagreement/disagree/ undecided/agree/total agreement). The age segment of the respondents was between 18 and 65 years.

RESULTS AND DISCUSSIONS

The developed questionnaire was completed by a number of 205 respondents. Following the processing of the answers in the section related to personal information, we can define the demographic variables in terms of gender, age, marital status, last form of education, presence of minors and the number of people in the household, employment status and monthly income. Analysing the obtained data, it can be seen that the survey participants are mostly women (76.1%) (Figure 1).

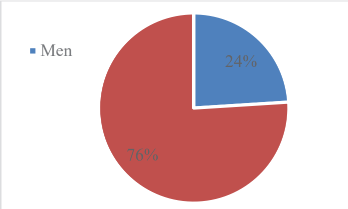

Figure 1. The gender of respondents

Regarding the distribution of the respondents according to gender and age, we can say that the majority segment is represented by 18-24 years old, both for women (40%) and men (13.17%) (Table 1).

Table 1. Distribution of respondents by age depending on gender

Age	Men	Women	Grand Total
18-24 years	13.17%	40.00%	53.17%
25-34 years	4.88%	11.22%	16.10%
35-44 years	2.93%	7.80%	10.73%
45-57 years	2.44%	11.71%	14.15%
58+ years	0.49%	5.37%	5.85%
Grand Total	**23.90%**	**76.10%**	**100.00%**

Table 2. Distribution of respondents by gender and marital status

Marital status	Men	Women	Grand Total
Married	4.88%	25.85%	30.73%
Unmarried	19.02%	50.24%	69.27%
Grand Total	**23.90%**	**76.10%**	**100.00%**

Also, from the marital status point of view, the majority of respondents (69.27%) are unmarried, respectively 50.24% women and 19.02% men (Table 2).
Regarding the distribution of respondents by gender and education, most of them (62.93%) have higher education (Table 3).

Table 3. Distribution of respondents by gender and study

Last school graduated	Men	Women	Grand Total
Vocational school/post-secondary school	0.00%	1.46%	1.46%
High school or less	8.29%	27.32%	35.61%
Higher education	15.61%	47.32%	62.93%
Grand Total	**23.90%**	**76.10%**	**100.00%**

With a percentage of over 72%, respondents declared that they have no children under the age of 18 in their household and approximately 28% of them have minors in their care. Regarding the number of people in the household, it can be observed that the highest percentage of 27.80% is represented by households with 2 people, followed by those with 3 and 4 people (Table 4).

Table 4. The presence of minor children and the number of people in the household

Minor children <18 years			
Row Labels	**Men**	**Women**	**Grand Total**
Yes	2.93%	24.88%	27.80%
No	20.98%	51.22%	72.20%
Grand Total	**23.90%**	**76.10%**	**100.00%**
Number of people in the household			
Row Labels	**Men**	**Women**	**Grand Total**
1	4.88	5.37	10.24
2	5.37	22.44	27.80
3	6.83	20.49	27.32
4	3.90	19.02	22.93
5	1.95	5.85	7.80
6	0.98	1.46	2.44
7	0.00	1.46	1.46
Grand Total	**23.90**	**76.10**	**100.00**

Table 5. The relationship between earned income and employment status

Row Labels	Full time (>40 h/week)	Part time (10-39 h/week)	Not working (inclusive <9 h/week)	Student
< 2000 RON	1.46%	2.44%	3.41%	10.24%
> 7001 RON	15.61%	0.49%	0.98%	4.88%
2001-3500 RON	8.78%	2.93%	1.95%	13.17%
3501-5000 RON	10.24%	0.49%	0.98%	6.83%
5001-7000 RON	9.27%	0.98%	0.49%	4.39%
Grand Total	**45.37%**	**7.32%**	**7.80%**	**39.51%**

Regarding the relationship between the employment status and income declared by the respondents (Table 5) it is highlighted the majority (45.37%) have a full-time job and among them a percentage of 15.61% achieve incomes over 7000 RON per month. Among the 39.51% students participating in the study, a percentage of 13.17% have an income between 2001-3500 RON per month.
Further, the results obtained after processing the responses of the 13 questions related to

consumption behaviour are presented. With a percentage over 82%, the respondents consumed in the last 12 months protein bars, dairy products with high protein content and protein-enriched snacks from vegetable sources, purchased from supermarket (Figure 2 and Figure 3).

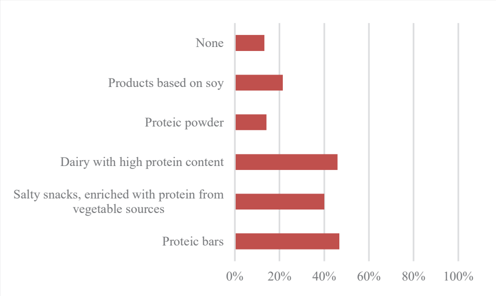

Figure 2. Products consumed in the last 12 months

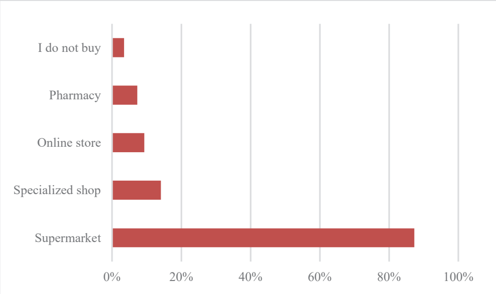

Figure 3. The place to buy salted protein snacks

The main source of information about salty snack-type products enriched with vegetable protein is the internet (~46%), followed by the selling place (34%) and family and friends (24.4%) (Figure 4).

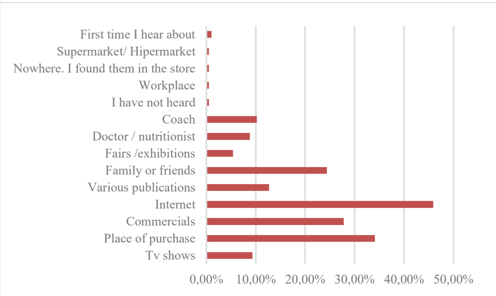

Figure 4. Information sources

Regarding the favourite combination of vegetables most wanted to be found in a salty snack product enriched with vegetable protein, the respondents have chosen mushrooms, peas, eggplant, asparagus and beans.

Further, most of the respondents (42.9%) consume vegetable, salty, protein-rich, minimally processed products occasionally, about 37% of them consume these kinds of products once per month or less often and 18% of the respondents consume them regularly (Figure 5).

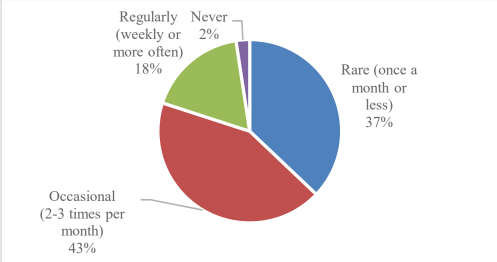

Figure 5. Frequency of consumption

Table 6 shows that regardless of age, 43% of respondents occasionally consume salty snack products enriched with vegetable protein, while 2% mentioned that they don't eat these types of products.

Table 6. Frequency of consumption of enriched products depending on age

Row Labels	18-24 years	25-34 years	35-44 years	45-57 years	58+ years	Grand Total
Never	3%	0%	0%	3%	8%	2%
Occasional (2-3 times per month)	43%	42%	41%	45%	42%	43%
Rare(once a month or less)	33%	42%	41%	41%	42%	37%
Regularly (weeklyor more often)	21%	15%	18%	10%	8%	18%
Grand Total	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**

A percentage of 36.6% of respondents fully read the information on product labels, 5.8% of them never read them, while 57.6% read the information partially (Figure 6).

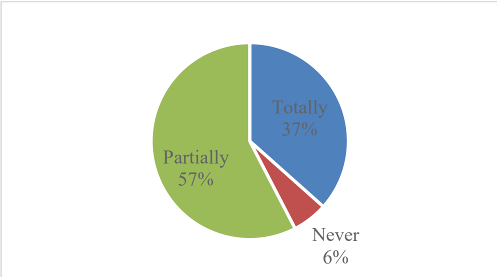

Figure 6. How the information on the label is used by the consumer

The distribution by age of the answers regarding the use of the information on the product label can be seen in Table 7. Regardless of age, more than half of the respondents, respectively 58%, partially read the information on the product label at the first purchase, while at the opposite pole (6%) are those who never read the label.

Table 7. Distribution of answers regarding the use of the label according to age

Row Labels	18-24 years	25-34 years	35-44 years	45-57 years	58+ years	Grand Total
Totally	28%	33%	68%	45%	50%	**37%**
Never	8%	3%	0%	3%	8%	**6%**
Partially	64%	64%	32%	52%	42%	**58%**
Grand Total	**100.00%**	**100.00%**	**100.00%**	**100.00%**	**100.00%**	**100.00%**

Correlating the level of education of the respondents with the desire for information on the purchased products, we can say that those with higher education read the information on the product label in percentage of 41% and partially in a proportion of 55% (Table 8).

Table 8. Distribution of answers regarding the use of the label depending on education level

Row Labels	Vocational school	High school	University	Grand Total
Totally	33%	29%	41%	**37%**
Never	0%	10%	4%	**6%**
Partially	67%	62%	55%	**58%**
Grand Total	**100.00%**	**100.00%**	**100.00%**	**100.00%**

Regarding the circular economy concept, a percentage of 57.6% of the respondents do not know it's definition, of which 47.8% are women and 9.76% are men (Table 9).

Table 9. Knowledge about the circular economy concept

Circular economy			
Row Labels	**Yes**	**No**	**Grand Total**
Men	14.15%	9.76%	23.90%
Woman	28.29%	47.80%	76.10%
Grand Total	**42.44%**	**57.56%**	**100.00%**

Even if the knowledge about circular economy concept is scarce between the respondents, 82.4% of them (of which 63.41% are women), are open to consuming products in which plant residues have been integrated (Table 10).

Table 10. Distribution of consumption of products in which vegetable residues have been integrated according to gender

Row Labels	Men	Women	Grand Total
Yes	19.02%	63.41%	82.44%
No	4.88%	12.68%	17.56%
Grand Total	**23.90%**	**76.10%**	**100.00%**

72.20% of the respondents, of which 56.10% were women, answered that they know that the food waste resulting from the use of vegetables and fruits (peels, seeds, sheaths, pulp, etc.) still contains valuable nutritional compounds such as vitamin C or antioxidants (Table 11).

Table 11. Information regarding the content of food waste

Row Labels	Men	Women	Grand Total
Yes	16.10%	56.10%	72.20%
No	7.80%	20.00%	27.80%
Grand Total	**23.90%**	**76.10%**	**100.00%**

For measuring the degree of acceptability of consumers towards vegetable salty snack-type products, with high protein content and nutritionally balanced, a multidimensional scale was used, and the respondents of this study had to express their agreement or disagreement with some statements of other consumers. In Table 12, the responses of the study participants are presented and processed.

For evaluating the degree of satisfaction of the respondents, the following calculation formula was applied:

$$\boxed{(G.S.\%) = \frac{\sum_{i,j} N_i \cdot P_j}{\sum N_i} \times 100}$$

where:

GS% - degree of satisfaction expressed as a percentage

N_i - the number of responses to the criterion i

P_j - the score assigned to each answer.

Table 12. The results of the study for the evaluation of the degree of acceptability

No.	Statements:	Totally disagree 1	Disagree 2	Indecise N/A 3	Agree 4	Totally agree 5	GS%
1	The consumption of vegetable products in which vegetable remains are integrated (peels, seeds, sheaths, pulp, etc.) resulting from the use of vegetables, contributes to the maintenance of human health	13	34	64	68	26	65.85
2	The consumption of such food products contributes to the protection of the environment	14	14	42	94	41	73.07
3	Vegetable, salty, protein-enriched food products are tastier than conventional products	7	13	32	82	71	79.22
4	For a healthy diet, I consume vegetable, salty, protein-enriched food products	18	38	61	61	27	64.00
5	I need more information about such products	12	34	94	50	15	62.15
6	The offer of vegetable, salty, protein-enriched, nutritionally balanced products from Romania is diversified	30	47	73	40	15	56.39
7	The quality/price ratio of these products is correct	19	19	38	101	28	69.76
8	Vegetable, salty, protein-enriched, nutritionally balanced products are recommended for athletes	32	68	78	19	8	50.54
9	I can't afford to buy such products	18	50	63	55	19	60.68
10	Some buy such products only because they are expensive	13	17	55	96	24	69.85
11	I tried such products out of curiosity	6	26	59	67	47	72.00
12	I have not heard of any campaign to promote these products	25	45	85	38	12	56.78
13	I get full faster when I consume such products	10	27	66	65	37	68.98
14	Such salty vegetable snack products with a high protein content are a quick alternative to main meals	7	21	67	73	37	70.93
15	Foods are recommended to be consumed in their integral form	19	39	68	62	17	61.85
16	The more a product is processed, the more it can lose vitamins, minerals or fibers from its composition	10	27	92	54	22	64.98
17	Vegetable products, salty, rich in proteins, nutritionally balanced are indicated in vegetarian or vegan diets	18	37	91	48	11	59.71
18	Consumers are paying more attention to the impact of food production and processing on health and the environment	11	17	53	44	80	76.10

CONCLUSIONS

Regarding the obtained demographic information, out of a total of 205 respondents, 156 (76.1%) were young women, over 50% of them aged between 18 and 34, unmarried, without minor children, graduates of higher education programs and coming from families consisting of a maximum of 4 people. Approximately 40% of the women participating in the study work full-time and their monthly net income is over 2000 RON. Related to consumption behavior, the respondents are open to new things, that is, they would consume a product in the composition of which food waste was integrated, respecting of course the rules regarding food safety, knowing that this resulting fruit and vegetable waste still contain valuable nutritional compounds. They also consume protein-enriched products 2-3 times a month, which they read about on the internet and buy from the supermarket. The respondents partially read the information on the label but would like to find mushrooms, peas, eggplant, asparagus and beans in the composition of such a product. The respondents agree that they contribute to environmental protection by consuming vegetable products in which vegetable residues are integrated and that these products are tastier than conventional ones. Some respondents consume such products out of curiosity and agree that they represent a quick alternative to meals. However, the respondents of this study do not fully agree that these products are not recommended only for athletes and in vegetarian diets. Furthermore, there isn’t a diversified offer of this kind of products and also there are no campaigns to promote them.

The GSM % value (average degree of satisfaction of the respondents) was of 65.71% and was calculated as the arithmetic mean of all GS% values obtained. This value shows satisfactory results regarding the 18 affirmations for which the respondents gave their opinions.

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ARTIFICIAL DIET AS AN ALTERNATIVE IN SILKWORM (*Bombyx mori* L.) FEEDING - A REVIEW

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Abstract

Silkworm (Bombyx mori L.) is an important monophagous insect in the sericulture industry. The mulberry leaves (Morus alba L.) are known as valuable plants rich in nutrients and nutraceuticals due to the presence of chemo-factors (morin, β-sitosterol) and antioxidants (flavonoids, anthocyanin and alkaloids), the traditional feed in the rearing of silkworm larvae. The mulberry is also used in pharmaceutical, food, beverage and healthcare industries, being considered a suitable plant for sustainable development. Over time, research on using the artificial diet in silkworms has been focused on different topics: i) rearing silkworms in the season when mulberry leaves are insufficient or not available and ii) as an organism model for human diseases (i.e., tumour, degenerative and metabolic diseases) due to the low breeding cost, short generation time, genetic background, large progeny size and numerous genes homologous to humans. This review aimed to summarize the literature information about the impact of nutrition as a key factor in silkworm rearing and the effectiveness of using an artificial diet based on different ingredients in silkworm productivity and health.

Key words: *artificial diet, nutrients, mulberry, silkworm (Bombyx mori L.)*

INTRODUCTION

Silkworm (*Bombyx mori* L.) is a remarkable monophagous, lepidopterous insect that occupies a special place within insect species suitable for several scientific studies. Their traditional feed consists of mulberry (*Morus* sp.) leaves (Saviane et al., 2014) that are more palatable compared to other vegetable leaves, selecting it as a preference due to the volatile compounds contained (Paudel et al., 2020). The rearing performance of silkworms and productivity depends on the nutritional composition and quality of mulberry leaves which usually are higher in the spring season than in autumn (Manjula et al., 2011; Lee and Choi, 2012; Zou et al., 2012; Elangovan et al., 2013; Chen et al., 2020; Qin et al., 2020). The other factors influencing mulberry leaves' nutritional and functional contents are cultivar, harvesting time, and the leaves maturity degree (Iqbal et al., 2012; Lee & Choi, 2012; Zou et al., 2012). However, Hu et al. (2013) noticed that the distribution of the leaves in the mulberry tree also affects the nutritional quality (the top leaves had higher nutritional contents than the mature leaves). Mulberry leaves have an excellent nutrient profile consisting of proteins, carbohydrates, sugars, lipids, vitamins, and microminerals (Zhou et al., 2015; Yu et al., 2018). Moreover, mulberry leaves are more capable of sequestering carbon (Hăbeanu et al., 2023). The bioactive compounds, i.e., flavonoids, polysaccharides, and alkaloids contained by mulberry leaves have hypoglycaemic, immunoregulatory, antioxidant, and anticoagulant properties (Wang et al., 2010; Devi et al., 2013; Wen et al., 2020; Zhang et al., 2022).
Conversely, the artificial diet quality did not change by season to mulberry leaves and may be used in germ-free rearing systems (Saviane et al., 2014). Although mulberry leaves are more attractive for *B. mori*, due to the fact that an artificial diet is pathogens-free, it is required when the silkworms are used as a biological model for various human diseases or as a bioreactor to obtain recombinant proteins and biomaterials based on silk (Kaito & Sekimizu, 2007; Kato et al., 2010; Tatemastu et al., 2012; Panthee et al., 2017; Paudel et al., 2020). Using invertebrates such as the silkworm as a biological model is more accessible due to the

short generation time, implies low-cost breeding, and does not require ethical approval (Panthee et al., 2017; Aznar-Cervante et al., 2021). This review aimed to summarize the literature information about the impact of nutrition as a key factor in silkworm rearing and the effectiveness of using an artificial diet based on different ingredients in silkworm productivity and health.

MATERIALS AND METHODS

For this review study, the relevant scientific articles with full-text available in English were searched in databases such as Web of Science, PubMed, ScienceDirect, Google Scholar, MDPI, Springer, and Elsevier by using the following keywords: "Artificial diet", "*Bombyx mori* L.", "*Morus alba* L.", "Mulberry leaf", "Nutrients", "Silkworm". We reviewed the relevant articles published over the last 20 years. References of included studies were also consulted as additional bibliographic sources.

RESULTS AND DISCUSSIONS

Nutrient requirements of silkworm

The chemical elements in the food necessary for the proper metabolism and growth of insects are known as their nutrient requirements (House, 1962). The correct balance of nutrients is crucial for most insects that have been studied (House, 1965). The nutrient requirements, i.e., carbohydrates, proteins, amino acids, fats, vitamins, minerals and as well as water, are commonly found in the mulberry leaves consumed by silkworms. The feeding strategy of silkworm larvae determines their subsequent development, cocoons and egg production (Shamsuddin, 2009).

Carbohydrates are a major energy source for silkworms (Ito, 1967) that can be produced from lipids and amino acids (Nation, 2001). Although some chemicals were used more quickly than others, silkworm larvae didn't show any particular requirements for carbohydrates, similar to other insect species. Pentoses often needed to be more utilized. By the hexoses examined, glucose, fructose, and mannose were efficiently used. Disaccharides were all of good quality, notably sucrose, cellobiose, and maltose. Melezitose and raffinose, two trisaccharides, had a high value (Ito, 1967). Sugar provides energy and carbon to silkworm larvae and stimulates their feeding. Among sugar alcohols, it was noticed that D-sorbitol promotes silkworm larva feeding (Ito, 1960 a, b). Sasaki et al. (2013) also reported improved silkworm larva feeding by adding myo-inositol and sucrose.

Proteins are fundamental substances in the body that plays a vital role in cell molecular structure and survival. Its ability to combine with other active substances, such as enzymes and hormones, allows it to regulate metabolism and physiological functions in silkworms. Around 70% of the silk proteins synthesized by silkworms are obtained directly from the protein present in mulberry leaves (Bhattacharyya et al., 2016). Adequate protein intake is essential for the development of ovaries and eggs in adult female silkworms, as it is necessary for the secretion of juvenile hormones. In contrast, male silkworms typically do not require protein to mature their sperm upon adulthood. Optimal proteins nutritional requirements vary by age, sex, physiological status and stress (Nation, 2001; Borah & Boro, 2020).

Amino acids (AA) are considered the most important components of silkworm nutrition. The silkworm larvae rely on the amino acids from mulberry leaves for their growth, development, and cocoon formation. The twelve essential AA for the growth and development of the silkworm larvae includes arginine, lysine, methionine, histidine, leucine, isoleucine, phenylalanine, threonine, tryptophan, valine, aspartic and glutamic acids (Borah & Boro, 2020). Besides this 12 essential AA, the silkworm must have semi-essential AA, proline. Meanwhile, alanine, cystine, glycine, serine, and tyrosine, known as non-essential AA, also play a significant role in promoting the growth of the silkworm. The addition of these non-essentials is necessary for optimal growth. Even when acidic AA were previously present in the diet, the nutritional effect of these non-essentials was still evident. The silk fibre fibroin includes alanine, serine, glycine, and tyrosine derived from the silkworm feed. Alanine, in particular, is crucial in metabolizing glucose, tryptophan, and organic acid (Borah & Boro, 2020).

Lipids are a group of compounds that include fatty acids, alcohols of varying chain lengths, steroids and their esters, phospholipids, and other similar substances. Silkworms have the ability to transform carbohydrates into lipids and store them in their body tissues (Nation, 2001). Fatty acids, phospholipids, and sterols are essential components of cell walls and perform various specialized functions. For normal growth and development, silkworm larvae require polyunsaturated fatty acids, particularly linoleic and linolenic acids (Genc et al., 2002). Mouths that lack these fatty acids experience wing formation defects, and their scales stick to the pupal case upon emergence. Even a small amount of lipids or sterols in their diet can positively influence the growth and development of silkworms.

Silkworms need sterols in their diet because they cannot synthesize enough to meet their physiological requirements. Sterols are present in all cellular membranes and serve as a precursor to the silkworm moulting hormone (Yuan et al., 2020). Therefore, a deficiency of sterols in their diet can result in an inability to moult, and silkworms typically die in their early stages (Nation, 2001; Genc et al., 2002). The addition of β-sitosterol is beneficial for the growth and development of silkworm larvae (Nagata et al., 2006).

Vitamins are organic compounds that predominantly serve as enzyme co-factors and catalysts. They regulate cellular metabolism and physiological functions (Shamsuddin, 2009). The growth and development of silkworms primarily depend on vitamin B complex and vitamin C intake. The main functions of these vitamins and their effect on silkworms are presented in Table 1 (Ito, 1978; Borah & Boro, 2020).

Table 1. Vitamins requirements for silkworms

Items	Role[1]	Minimum req. mg/g of dry diet[2]	Mulberry leaves content mg/g dry matter[2]	Effect on silkworm[1]
Vitamin C (ascorbic acid)	- antioxidant; - protection against oxidative damage to DNA, membrane lipids and proteins.	-	-	- addition of 1-2% vit. C increases the weight of larvae and survival rate; - absence of vit. C in the instars I and II delayed the growth.
Vitamin B1 (thiamine)	- energy metabolism.	0.5	6.7	- no effect on larvae weight and silk gland; - increase larval duration, cocoon and shell weights, and fecundity.
Vitamin B2 (riboflavin)	- energy released from carbohydrates, fats, and proteins.	5	13-21	- increase certain economic traits and improve silk production.
Vitamin B3 (niacin)	- cell respiration; - release of energy and -metabolism of carbohydrates, lipids and proteins.	20	69-99	-higher vit. B3 dose affects feed intake, growth, increased mortality in the moulting phase, and incomplete moulting.
Vitamin B5 (pantothenic acid)	- cofactor for enzymes; - precursor of coenzyme A; - metabolism of carbohydrates; - synthesis and degradation of fats; - synthesis of sterols and steroid hormones.	5	16-35	
Vitamin B6 (pyridoxin)		20	43-50	- lower vit. B6 dose increases the larval body weight, pupal weight, silk gland weight, cocoon and shell weight. - higher vit. B6 dose affects the optimum growth and development of the silkworm.
Vitamin B8 (biotin)	- carbohydrate and fat metabolism, - synthesis of fatty acids.	1	0.2-0.8	- minimal optimal level of vit. B8 for growth and survival of the silkworm is much lower than those of other vitamins.
Choline and Inositol	- production of cell membranes.	750 1000	930-1550 4000	- need a higher level for optimal growth.

Sources: [1]Borah and Boro (2020); [2]Ito (1978).

Minerals act as a limiting factor for the growth of insects, which is particularly true for all types of dietary compositions (Ito, 1978). Salt significantly enhanced the growth of developmental stages, improved cocoon characteristics, triggered early cocoon production, and increased the reproductive potential of silkworms. Dietary supplementation with nickel chloride, potassium iodine, and copper sulphate elevated the economical parameters of silkworms. Nickel chloride boosted the growth of silkworm larvae, pupae, and adults, as well as cocoon production, but higher concentrations of salt had adverse effects on these parameters (Ito & Nirminura, 1966). Feeding silkworm larvae with mulberry leaves fortified with nickel and zinc increased cocoon weight (Wright, 1984).

Chemical composition of mulberry leaves

To cover the required nutrients of *B. mori* and produce artificial diets suitable for healthy and adequate growth, it is necessary to know the nutritional value starting with mulberry leaves, considered an excellent functional feed.

The chemical composition of mulberry leaves, fresh and meal, according to the literature database (Heuze et al., 2019 - www.feedipedia.org), is given in Table 2.

Crude protein, fat content and gross energy value were higher in fresh mulberry leaves than in meal, while crude fibre and its fractions, ash, and condensed tannins were lower in fresh mulberry leaves versus meal. Proteins are the most prevalent nutrient in leaves that larvae take over and are found in the pupae composition (Hăbeanu et al., 2023). Practically, protein and their AA from mulberry leaves are converted into silk.

Table 2. Proximate composition of mulberry leaves (*Morus alba*)[1]

Items	Mulberry leaves	
	fresh	meal
Dry matter (DM, % as fed)	30.2	90.5
Crude protein (% DM)	19.1	18.0
Crude fat (% DM)	5.6	3.5
Crude fibre (% DM)	13.5	13.7
Neutral detergent fibre (% DM)	30.9	37.0
Acid detergent fibre (% DM)	22.3	25.1
Ash (% DM)	12.3	12.8
Lignin (% DM)	5.4	6.1
Condensed tannins (% DM)	7.0	30.0
Gross energy (MJ/kg DM)	18.2	17.5

Source: [1]Heuze et al. (2019) - www.feedipedia.org

Fresh mulberry leaves have an excellent AA profile (Table 3) with a total of 80.6 g/16 g N, from which 38 g/16 g N essential AA and 42.6 g/16 g N non-essential AA. The essential/non-essential AA and essential/total AA ratios represent 0.89% and 0.47%, respectively (Heuze et al., 2019 - www.feedipedia.org). Olteanu et al. (2015), expressing the AA profile of mulberry leaves meal at % DM, reported 19.43% total AA, from which 19.15% essential AA and 10.28% non-essential AA. The essential/non-essential AA and essential/total AA ratios represent 0.85% and 0.47%. Amino acids are protein building blocks and function as signalling molecules that control feed intake, protein phosphorylation, gene expression, and intercellular communication (Yang et al., 2018). The primary AA in silk fibroin are glycine, serine, alanine, and tyrosine (Mondal et al., 2007). According to Qin et al. (2020), the decreased levels of glycine, serine, alanine, and tyrosine in an artificial diet could affect the biosynthesis of silk protein in silkworm larvae. Therefore, AA supplementation of artificial diet may enhance silkworm larvae feeding efficiency (Qin et al., 2020).

The functional characteristics of mulberry leaves are also given by their valuable fatty acids (FA) composition (Hăbeanu et al., 2023), such as myristic, palmitoleic, oleic, octadecatrienoic, octadecadienoic, hexadecenoic and octadecanoic acids (Horie et al., 1985). Olteanu et al. (2015) found that the mulberry leaves meal contains 36.68% of fat, saturated FA, 13.60% monounsaturated FA, and 44.11% polyunsaturated FA (PUFA), from which n-3 PUFA represents 29.27%, and n-6: n-3 ratio 0.51% (Table 4).

Table 3. Amino acids profile of mulberry leaves (*Morus alba*)

Items	Mulberry leaves	
	fresh[1] (g/16 g N)	meal[2] (% DM)
Lysine	4.2	1.68
Methionine	1.9	0.34
Threonine	3.9	1.53
Arginine	7.4	1.13
Valine	5.6	0.85
Phenylalanine	3.8	0.98
Isoleucine	3.6	0.91
Leucine	7.6	1.73
Essential AA[3]	*38.0*	*9.15*
Aspartic acid	8.8	3.27
Glutamic acid	8.9	2.93
Alanine	5.1	1.39
Glycine	4.8	0.86
Histidine	2.2	-
Proline	4.4	-
Serine	4.6	1.14
Tyrosine	3.8	0.69
Non-essential AA[3]	*42.6*	*10.28*
Total AA[3]	80.6	19.43
Essential/Non-essential AA ratio[3]	0.89	0.85
Essential/Total AA ratio[3]	0.47	0.47

Sources: [1]Heuze et al. (2019) - www.feedipedia.org; [2]Olteanu et al. (2015); [3]Calculated values.

Table 4. Fatty acids profile of mulberry leaves (*Morus alba*)[1]

Items (% fat)	Mulberry leaves
	meal
Myristic acid (C14:0)	1.37
Pentadecanoic acid (C15:0)	2.42
Palmitic acid (C16:0)	25.22
Heptadecanoic acid (C17:0)	0.44
Stearic acid (C18:0)	5.36
Arachidic acid (C20:0)	1.87
Saturated FA (SFA)[2]	*36.68*
Myristoleic acid (C14:1)	0.64
Pentadecenoic acid (C15:1)	7.24
Palmitoleic acid (C16:1)	2.35
Oleic acid (C18:1n-9)	3.37
Monounsaturated FA (MUFA)[2]	*13.60*
Linoleic acid (C18:2n-6)	13.50
α-linolenic acid (C18:3n-3)	29.07
Octadecatetraenoic acid (C18:4n-3)	0.20
Eicosatrienoic acid (C20:3n-6)	0.81
Arachidonic acid (C20:4n-6)	0.53
Polyunsaturated FA (PUFA)[2]	*44.11*
Σ n-6 PUFA[2]	14.84
Σ n-3 PUFA[2]	29.27
n-6: n-3 ratio[2]	0.51

Sources: [1]Olteanu et al. (2015); [2]Calculated values.

The minerals composition provided in Table 5 revealed that in mulberry leaves, the main macro-mineral was calcium, followed by potassium, magnesium, phosphorus and sodium in both fresh and meal form, whereas iron was the main micro-minerals, followed by the zinc, manganese and selenium in fresh mulberry leaves (Heuze et al., 2019 - www.feedipedia.org).

Table 5. Minerals composition of mulberry leaves (*Morus alba*)[1]

Items	Mulberry leaves	
	fresh	meal
Macrominerals (g/kg DM)		
Calcium	22.3	42.3
Phosphorus	3.2	4.2
Potassium	17.5	21.7
Sodium	2.0	1.2
Magnesium	4.9	4.7
Microminerals (mg/kg DM)		
Manganese	31	-
Zinc	55	-
Cooper	10	-
Iron	322	-
Selenium	0.10	-

Source: [1]Heuze et al. (2019) - www.feedipedia.org

The vitamins composition of mulberry leaves contains 14.0 mg/100 g β-carotene, 11.30-15.37 mg/100 g vitamin C, 0.58 mg/100 g vitamin B2, and 0.04 mg/100 g vitamin B3 (Khakwani et al., 2022), while Dhiman et al., (2020) reported 13.6 mg/100 g β-carotene, 15.20-24.42 mg/100 g vitamin C, 0.36 mg vitamin B2, and 0.88 mg/100 g vitamin B3. Mulberry leaves contain as the main active compound 1-Deoxynojirimycin (DNJ), known for its higher α-glucosidase inhibitory activity that suppresses postprandial blood glucose, prevents diabetes mellitus and decreased lipid accumulation (Tsuduki et al., 2009; Asai et al., 2011; Li et al., 2013). Several studies have found that the DNJ concentration of different mulberry leaves varieties varied from 0.08 to 1.12 mg/g dry weight (Hu et al., 2013; Yu et al., 2018) or from 0.03 to 1.7 mg/g dry weight (Vichasilp et al., 2012).

The bioactive compounds concentrations of mulberry leaves determined by Khakwani et al. (2022) were 660 mg/100 g alkaloids, 226 mg/100 g rutin, 763 mg/100 g quercetin, 432 mg/100 g catechins, 33.89 mg/100 g total flavonoids, 12.26 mg/100 g total phenols, and 0.2 mg/100 g hemicellulose. These bioactive compounds possess health-promoting effects such as antioxidant, antibacterial, anticancer, antidiabetic, hepato-, cardio-, neuro-protective, antihypertensive, and antiinflammatory antiapoptosis, antiarteriosclerosis, antiviral, and antidepressant properties (Hassan et al., 2020; Wang et al., 2022). Moreover, the phytochemicals compounds from various mulberry plant components, including the fruits (caffeic acid, catechin hydrate, chlorogenic acid; Yuan and Zhao, 2017), roots (kuwanon S, mulberry side A, mulberry side C, cyclomorusin; Singh et al., 2014), and woods (chlorogenic acid, maclurin, oxyresveratrol; Ahn et al., 2017) were also studied for their antioxidative, antidiabetic, antiinflammatory and antitumor biological properties (Dhiman et al., 2020).

Artificial diet composition

Horie (1995) stated that the artificial diets for silkworms required at least three conditions: i. to cover the nutritional requirements of silkworms; ii. to have adequate physical characteristics; iii. do not contain contaminants; iv. do not contain substances which can affect silkworms.

The artificial-based diet in silkworm-rearing technologies has been developed by researchers in Japan and China (Cui et al., 2016, cited by Dong et al., 2017). Japan applied the artificial-based diet in silkworm rearing from the 1980s to the 1990s at a large-scale (Hamamura, 2001, cited by Dong et al., 2017). Still, in China, the largest sericulture country in the world, artificial diet silkworm-rearing needs to be implemented (Dong et al., 2017).

In Bulgaria, Tzenov and Georgiev designed in 2010 an artificial diet that could be used during the entire larval cycle and for all-year seasons (Avramova et al., 2020).

The artificial diet formulation was based on the nutrient profile of mulberry leaves as the “gold standard food” (Qin et al., 2020). The formulation of optimal silkworm diets involves several practical issues and considers that the nutritional requirements depend on the silkworm's productivity (Cappellozza et al., 2005).

The composition of the artificial diets is based on different amounts of dried mulberry leaf powder, defatted soybean meal, wheat meal, corn starch, soybean fibre, ascorbic acid, citric acid, vitamin mixture, salt mixture, agar, sorbic acid, propionic acid, chloramphenicol and β-sitosterol (Cappellozza et al., 2005). According to Bhattacharyya et al. (2016), the dry components of an artificial diet are mixed with antibiotics (chloramphenicol or dihydro-streptomycin) or other substances (ascorbic or propionic acids) that have anti-microbiological activity and support the developmental cycle of silkworms. Even over time, the artificial diet formulae for silkworms were improved, and the metabolic utilization of the artificial diet was lower compared to mulberry leaves (Dong et al., 2017).

Studies have shown that several productive silkworm breeds, which initially had a low response to artificial diet, were gradually adapted to it through selective breeding across generations. Trivedy et al. (2001; 2003) created five multi- and six bivoltine strains that readily accepted the artificial diet and exhibited favourable economic characteristics comparable to those of their counterparts raised on mulberry leaves.

Avramova & Grekov (2013) conducted trials on hybrids, and due to the confirmed resilience of silkworm to breeding with artificial diet, they proposed formulating diets with different levels of dried mulberry leaves.

It has been stated that the silkworms can detect the mulberry odour and are drawn to β-γ-hexenol and α-β-hexenal elements present in the mulberry. This scent is perceived by the GR66 gene, which encodes a hypothetical gustatory receptor that is believed to be bitter, that are responsible for the silkworms' particular feeding preference for mulberry (Zhang et al., 2019).

Table 6 shows some examples of artificial diet composition used in silkworms *B. mori*.

Table 6. Examples of artificial diet composition for *B. mori* silkworms

Ingredients	Reference
30% mulberry leaves powder, 28% defatted soybean meal, 15% cellulose powder, 6.1% corn starch, 3.7% citrate, 4% salt mixture, 4% sucrose, 7% agar, 0.4% ascorbic acid, 0.4% vitamin B mixture, 0.3% phytosterol, 1.3% soybean oil refined, 1.3, and 1% antiseptic.	Shinbo and Yanagaw (1994)
54.6% dried tofu cake, 25% defatted soy bean powder, 15% dried mulberry leaf, 2% citric acid, 1% ascorbic acid, and 2.4% others.	Sasaki et al. (2000)
25 g dried mulberry leaf powder, 36 g defatted soybean meal, 15 g wheat meal, 4 g corn starch, 5 g soybean fibre, 4 g citric acid, 2 g ascorbic acid, 3 g salt mixture, 4.2 g agar, 399 mg vitamin mixture, 200 mg sorbic acid, 691 mg propionic acid, 10 mg chloramphenicol, and 500 mg β-sitosterol per 100 g dry weight.	Cappellozza et al. (2005)
28% mulberry powder, 25% soya powder, 4% salt mixture, 0.3% sterol, 3% sugar, 3% cellular power, 7% jellying agent, and 3% preservative.	Rajaram et al. (2012)
36 g dried mulberry leaf, 30 g defatted soybean meal, 4 g wheat meal, 4 g rice meal, 4 g corn starch, 4 g citric acid, 2 g ascorbic acid, 3 g salt mixture, 8 g agar, 1 g aloe vera gel, 4 g potato starch and water.	Bhattacharyya et al. (2017)
35% mulberry leaf powder, 35% soybean powder, 15% green twig powder, 9.4% starch, 1.5% vitamin C, 1.5% vitamin B complex, 2% citric acid, 0.4% crotonic acid, and 0.2% choline chloride.	Dong et al. (2017) Dong et al. (2018)
20 g mulberry powder, 0 g soybean flour, 2.5 g agar/100 ml water. 16 g mulberry powder, 4 g soybean flour, 2.5 g agar/100 ml water. 10 g mulberry powder, 10 g soybean flour, 2.5 g agar/100 ml water. 4 g mulberry powder, 16 g soybean flour, 2.5 g agar/100 ml water. 0 g mulberry powder, 20 g soybean flour, 2.5 g agar/100 ml water.	Paudel et al. (2020)

Effect of using the artificial diet in silkworm

As it provides adequate nutrition and disease-free conditions throughout the year, silkworm raising on the artificial diet has numerous benefits over current practices; the first, second, and third instars are crucial for this (Nair et al., 2011). Sasaki et al. (2000) evaluated the effect of silkworm diets (artificial versus mulberry leaves) on the raw silk proteins dyeing properties. The authors found higher sericin levels, higher dye uptake and slower dyeing rate in raw silk samples, while silk fibroin exhibited similar equilibrium dye absorption and dyeing speed as effect of fed artificial than mulberry leaves.

Cappellozza et al. (2005) studied the effects of the artificial diet without or with 2% vitamin C on *B. mori* silkworm larvae throughout larval life or only in some larval instars. These authors reported that complete vitamin C deprivation during the larval cycle affects larval growth and cocoon production. Moreover, vitamin C deprivation from the diet during the first and last larval instars has positively affected cocoon production without influencing the mortality rate or delaying the larval cycle.

Tzenov & Georgiev (2010), cited by Avramova et al. (2020), noticed that using an artificial diet with a content of 38% mulberry meal shorter the silkworm larvae period during the fifth instar of the whole rearing period without altering the other production characteristics when compared to silkworm reared on mulberry leaves.

Nair et al. (2010) highlighted the possibility of using chawki rearing to produce breeding resource material for potential hybrids of multivoltine silkworms, using only artificial feed.

Several studies reported that feeding a semi-synthetic artificial diet (SeriNutrid) in silkworm larvae up to chawki level did not affect the economical parameters of silkworm (Rajaram et al., 2012), or even increased the cocoon and shell weights, or shell ratio, thus obtaining a higher price (5-10%) for premium quality cocoons (Mondal et al., 2018).

Bhattacharyya et al. (2017) evaluated the antioxidant activity of artificial diet ingredients, i.e., defatted soybean meal, rice meal, wheat meal, corn starch, potato starch and Aloe vera gel along with mulberry leaf. These authors found that soybean has a higher impact on increasing cocoon shell weight and silk quality than mulberry leaves.

Avramova et al. (2020) reported that the silkworms reared on an artificial diet during the summer season in their first three instars, and mulberry leaves in their four and five instars obtained similar technological characteristics and optimal sanitary conditions compared to silkworms reared only on mulberry leaves.

Moise et al. (2020) fed different *B. mori* silkworm breed with an artificial diet with addition of 1% or 5% bee pollen noticed an insignificant improvement in biological parameters, especially in the case of 5% bee pollen addition.

Until now, studies on artificial diets have focused on improving silk production and quality. In the current context of rising interest in employing silkworms as drug discovery models, an artificial diet that is straightforward, usable by a large population, and suitable for the study is preferred. Several studies have also been conducted comparing the intestinal microbiota of silkworms fed on natural and artificial diets and their proteomic and metabolomic (Dong et al., 2017; Dong et al., 2018) profiles. The diversity of gut microbiota and the primary bacteria present in silkworms raised on an artificial diet exhibited significant variations as compared to those raised on mulberry leaves. This dissimilarity could be associated with factors such as growth, metabolic processes, and the ability to resist diseases in silkworms fed an artificial diet (Dong et al., 2018). Moreover, the alterations in the gut microbiota could also affect the silkworms' nutrient metabolism and immune resistance, which may be attributed to their adaptation to the long-standing evolutionary practice of consuming mulberry leaves (Dong et al., 2018).

When compared the metabolomics of silkworms raised on fresh mulberry leaves versus artificial diets, Dong et al. (2017) observed that the silkworms reared on artificial diets faced a severe deficiency of certain vitamins and experienced down regulation of glycolysis, the TCA cycle, and lipid metabolism. However, these larvae showed increased levels of various amino acids and amino acid-related metabolites. Dong et al. (2017) also stated that focusing on the balance of amino acids and developing a more efficient method for vitamin supplements in artificial diets could result in significant advancements.

Paudel et al. (2020) tested five simple artificial diets based on different amounts of soybean flour and mulberry leaf powder and the same agar content to screen drug candidates in silkworm hyperglycaemic and infection models. The authors concluded that using silkworms for biological, biotechnological, and pharmacological investigations depends on the accessibility of a simple artificial diet for feeding trials (Paudel et al., 2020). Comparing the fecal metabolome of silkworms fed mulberry leaves versus artificial diet, Qin et al., (2020) have shown that the silkworms raised on mulberry leaves increased the concentration of amino acids, carbohydrates, and lipids, which are essential for physical growth and silk protein synthesis. Conversely, the silkworms fed on artificial diets had a relatively higher level of urea, citric acid, D-pinitol, D-(+)-cellobiose, and N-acetyl glucosamine (Qin et al., 2020).

CONCLUSIONS

Based on the literature information available, it can be concluded that nutrition plays an essential role in silkworm and mulberry leaves, as traditional silkworm feed sources are valuable plants rich in nutrients (carbohydrates, protein, amino acids, fatty acids, minerals, vitamins), as well as nutraceuticals (morin, β-sitosterol) and antioxidants (flavonoids, anthocyanin and alkaloids). The present review highlighted that the effectiveness of an artificial diet on silkworms' productivity and health depends on the diet composition (especially the % of mulberry leaves and other protein sources). Though there is debate regarding the artificial diet's efficacy, it nevertheless attracts interest, particularly for research using models from the medical field. The artificial diets must give essential nutrients to meet the needs of the silkworms' larvae to have better-suited qualities. Due to the employment of silkworms as research models, it is necessary to include various compounds in artificial diets to explore specific mechanisms.

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RED YEAST β-CAROTENE CONTENT: DEVELOPING EXTRACTION AND DETERMINATION FOR IMPROVING POULTRY NUTRITION

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Abstract

Vitamin A is an essential nutrient, for both production and reproduction of farm animals, however, most animals are unable to synthesize de novo precursors of vitamin A (β-carotene), and dietary supplementation is mandatory. The current paper aims to evaluate the analytical parameters and to improve the method efficiency for determining β-carotene content in red yeast and the internal laboratory validation protocol. Our method stands for the ultraviolet-visible spectrophotometric uses, having the Lambert-Beer law as the basis. To determine the β-carotene content, dimethyl-sulfoxide was employed as a solvent, calibration curve, and visible spectra were evaluated (300-900 nm). The linearity of beta carotene measured at 465 nm using the UV-Vis method linearity range was 6.1-36.6 μg/ml, R^2=0.997, LOD = 5.39 μg/ml, LOQ = 16.32 μg/ml, SD less than 5% and RSD in between 1-15%. In conclusion, the β-carotene spectroscopic method determination is a cheap, and efficient method, suitable for β-carotene determination and retinol and retinoic acid estimation for nonconventional feed additives such as yeasts.

Key words: *β-carotene, retinol, spectrophotometry, UV-Vis method.*

INTRODUCTION

Red yeasts can synthesize carotenoid complex (Frengova & Beshkova, 2009) and might be an alternative to synthetic vitamin and pigment dietary additives used in livestock (Marounek & Pebriansyah, 2018; Meléndez-Martínez et al., 2022). Recent interest in the utilization of microbial β-carotene sources has been stimulated by reproductive performance in poultry (Sajjad et al., 2020), but also by the meat and egg quality improvement when the dietary supplementation took place (Kanwugu et al., 2021; Sun et al., 2020). Poultry farming is an important branch of agriculture in many countries and the main challenge is driven by the organic farms producing units, unable to utilize chemical additives (Pailliè-Jiménez et al., 2020; Pandey & Kumar, 2021), and organic diets are mandatory. In poultry physiology, carotenoid synthesis is absent and the exogenous dietary intake is essential (Ortiz et al., 2021), for further conversion of both xanthophylls and retinal/retinoic acid. Common farming practice employing chemically developed formulas of carotenoids as pigment additives (Ribeiro et al., 2018), for example, β-Apo-8′-carotenal, or
canthaxanthin (Surai & Kochish, 2020) is often a conventional dietary strategy for both broiler chicks (Grashorn, 2016) and laying hens (Ortiz et al., 2021), in order to supplement the nutritional requirements (broilers - 10000 IU vitamin A, and laying hens 6500 IU vitamin A) for improving product quality attributes (ISA, 2020; NRC, 1994).
The study of the chemical composition, and the determination of the complex of carotenoids, in the matrix of non-conventional raw materials, is an important step in their potential nutritional use. Considering the fact that the complex carotenoid matrix presents in yeast such as *Rhodotorula mucilaginosa* is directly influenced by the strain genetic determinism (Pino-Maureira et al., 2021) and productivity also modulated by both yeast phenotype (Zhang et al., 2016) and the engineering approach via metabolites enhancement (Verma et al., 2019). Furthermore, adapting and improving the in-processes followed by

quantitation and qualifications of productivity to obtain product maximization with the minimal economic implication is a novel trend.

UV-Vis (UV-visible spectrophotometry) is a spectroscopic approach, absorption or reflection spectroscopy in the ultraviolet part and the complete adjacent regions of the electromagnetic spectrum (Popescu et al., 2022). It is a relatively cheap and easy-to-use instrumental application, also this methodology is widely used in various fundamental connected domains. Absorption spectroscopy is complementary to fluorescence spectroscopy.

The paper focuses on the chemical instrumental method extraction and development of yeasts carotenoids, while improving the dietary carotenoid dosage retinoic acid estimation for poultry nutrition.

MATERIALS AND METHODS

The method development basis was previously described by (Barba et al., 2006), employing hexane and acetone as extractive complex solvents.

In order to develop the instrumental UV-Vis method of yeasts carotenoids, the following reagent, and reference standard were used: dimethyl sulfoxide (DMSO, BioReagent, suitable for hybridoma, ≥99.7%, Sigma-Aldrich, Missouri, USA), type I β-carotene reference standard (synthetic, ≥93%, suitable for UV determination, powder Sigma-Aldrich, Missouri, USA), ultrapure water (chromatographic resistivity at least 18,4 MΩ, and having the total organic substances max. 29 ppb). Previously, three batches of *Rhodotorula mucilaginosa* biomass were optimized (nutritive substrate, on an orbital shaker New Brunswick Scientific, Innova40®, Germany) for biomass yield and biomass carotenoid complex (unpublished data). For the carotenoid extraction, the yeast biomass was previously lyophilized at -50 °C, at 0.370 mBar (Labconco freeze dryer, Kansas, USA) and weighed on an analytic balance (Precisa XT220A, Switzerland).

Analyte extraction

The 96-h fermented *Rhodotorula mucilagionosa* lyophilized biomasses (3 replicas/batch) were individually resuspended in 2.5 ml of DMSO, ultrasonicated for 15 minutes (Elmasonic S 50 R, Germany), and centrifugated at 4°C, 10000 rpm, during 5 minutes (Sigma 2-16PK refrigerant centrifuge, Germany). The collected yeast pellets were washed (as mentioned before), during four successive cycles, finally collecting the amount of 10 ml supernatant/per each sample washed.

Reference standard preparation

The β-carotene stock solution was prepared, by solubilization in DMSO, having a concentration equal to 366.66μg/ml. The working standard solution was prepared freshly, in DMSO, and spiked (6.1, 9.15, 12.2, 15.5, 18.3, 21.35, 24.4, and 36.6 μg/ml), for further efficacy, a standard etalon curve was developed.

In order to determine the analytical recovery and method precision, the amount of β-carotene present in each yeast's samples were calculated, using the linear regression equation, after developing the standard curves, just by taking into consideration the absorbance (optical density, OD values). Triplicate determinations were employed for each sample, and at the end was calculated the percentage of the recovery. For all three yeast batches samples, were calculated the standard deviations (SD) and the relative standard deviations (RSD), by analysing the minimum, maximum, and average concentrations, three sets for each experimental batch, when compared with the blank.

Selectivity

The ability of a method to identify particular analyte) in a complicated mixture without becoming interfered with by other mixture constituents is referred to as selectivity. Specific means that a procedure is completely selective for a given analyte or group of analytes (Verbić et al., 2013).

For determining the method specificity, chemical (NaOH 0.1N and HCl 1M), and physical-chemical (heat stress - exposed during 2h, and 3h at 40°C) processes were employed, for influencing the analyte structure in the reference solution (36.6 μg/ml) and product tests solutions (5.22, 5.31, and 4.73 μg/ml), in triplicate. RS solution: 3.66 mg of standard reference was diluted to a balloon of 10 ml with

DMSO. TS solutions: 10 ml of supernatant collected after washing the yeast pellets with DMSO. Blank: 10 ml DMSO. Stress 1: 2 ml of TS, and 0.5 ml of 0.1N NaOH, diluted to balloon 10 ml, with DMSO. Stress 2: 2 ml of TS, and 0.5 ml of 1M HCl, diluted to balloon 10 ml, with DMSO. Stress 3: 2 ml of TS, diluted to 10 ml DMSO, and exposed for 2h at 40°C drying oven (Binder, ROTH, Germany). Stress 4: 2 ml of TS, diluted to 10 ml DMSO, and exposed for 3h at 40°C drying oven (Binder, ROTH, Germany).

Each stress test solution (n = 24) spectra were evaluated for maximum absorbance read, and overlaying characteristics.

Linearity

The capacity of an analytical process to produce test results that are directly proportional to the concentration (quantity) of analyte in the sample is known as linearity (Guy, 2014). The linear concentration range and the assay variability, mandatory imply the development of the limit of detection and the limit of quantification. The lowest quantity or concentration of a component that can be consistently detected using a particular analytical procedure is commonly referred to as the limit of detection (LOD). The smallest amount or lowest concentration of a material that can be determined using a certain analytical process with the established accuracy, precision, and uncertainty is referred to as the limit of quantification, or LOQ.

$$LOD = \frac{3.3*\sigma}{S},$$
$$LOQ = \frac{10*\sigma}{S},$$

having the σ – the standard deviation of the response, and S – the slope of the calibration curve. Stock solution: RS solution: 3.66 mg of standard reference was diluted to a balloon of 10 ml with DMSO. Linearity solution 6.1 ppm: 50µl TS to 3 ml DMSO. Linearity solution 9.2 ppm: 75µl TS to 3 ml DMSO. Linearity solution 12.2 ppm: 100 µl to 3 ml DMSO. Linearity solution 18.3 ppm: 150 µl to 3 ml DMSO. Linearity solution 36.6 ppm: 300 µl to 3 ml DMSO. Reading in triplicate each linearity solution, calculating the β-carotene mean absorption value, SD standard deviation (%), and RSD (%) relative standard deviation.

Precision

The degree of agreement among individual test findings when the method is performed on several samplings of a homogenous sample is defined as the precision of an analytical method. The standard deviation or relative standard deviation (coefficient of variation) of a sequence of measurements is commonly used to express the precision of an analytical technique (Guy, 2014). Often, the method precision attribute is known as repeatability. For this assay, two analysts were employed, preparing a similar sample solution for determining the β-carotene content present in the 36.6 ppm solution. Sample preparation: 36.6 ppm β-carotene in DMSO. The simple solutions were evaluated six-time, saving the maximum value of absorption, wavelength (465nm) for calculating the mean values, the SD, and the RSD (%) ≤ 5%.

Statistical data

All data were analysed using the XLSTAT for Excel 2021 version software (Addinsoft, New York, USA).

RESULTS AND DISCUSSIONS

Selectivity

Stress solutions and reference standard solutions spectra (300-900 nm) were developed by using the quartz 1cm cuvettes (Figure 1). The percentage of the analyte recovery is displayed in Table 1. The lower results were obtained when the red yeast sample solution was exposed to heat, for 2 h (82.32 ± 3.45), and 3 h (74.56 ± 3.57), when compared with the reference solution. Similar to our findings, suggests that heat influenced the β – carotene present in food (Borba et al., 2019) and feed (Thakur, 2018).

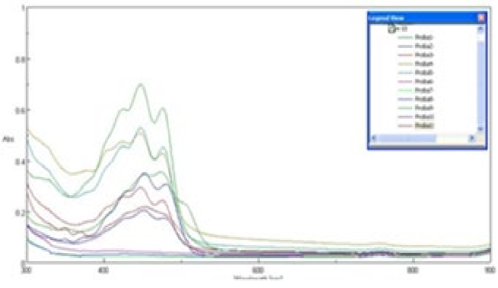

Figure 1. β-carotene in DMSO method selectivity

Our method possesses the selectivity attribute, thus the analyte effective separation. At the same time, no peaks were detected, indicating that our stress solutions are not interfering with the β-carotene assay, and could not influence the determination. Furthermore, the acceptance criteria implied the efficient separation of β-carotene from the red yeast matrix, under different conditions. The maximum absorption was at 465 nm, specific for the β-carotene standard reference (Chábera et al., 2009).

Table 1. Stress solution β-carotene content variations

Analyte recovery	0.1N NaOH	1M HCl	2h40°	3h40°
Mean	97.64	101.5	82.32%	74.56
SD	3.42	2.38	3.45	3.57
RSD	2.76	3.23	3.88	4.22

Linearity

A linear regression graphic was developed, having the general equation y = ax + b, the abscissa originating 0, indicating the linear solution concentration and the ordinate axis, resulting in the linear solution absorption. For validating the instrumental method linearity, the regression coefficient ($R^2 \geq 0.997$) was calculated, having the RSD %, less than 5%.

In Table 2 and Figures 2 and 3 the method's linearity assay results are displayed.

Table 2. Test solutions recovery percentage

Test solution	Mean value	SD	RSD (%)
6.10 ppm	0.0479	0.00090185	1.88015973
9.15 ppm	0.0669	0.00065064	0.98185217
12.20 ppm	0.0841	0.00110942	1.30087467
18.30 ppm	0.1090	0.00041633	0.38242486
36.60 ppm	0.1280	0.00100000	0.78740157

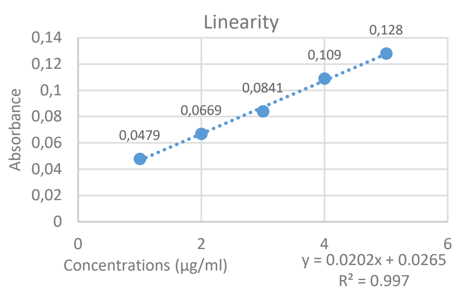

Figure 2. β-carotene linear range

Our results indicate that the studied concentration range (6.1-36.6 ppm) is linear, with 99.7% of accuracy, and the LOD and LOQ values are corresponding to the EPh. requirements (Bouin & Wierer, 2014), having LOD's signal-to-noise ratio, S/N≥3, and LOQ's ratio S/N≥10. The RSD is less than 5%, and the R2 is higher than 99.7% for the β-carotene assay.

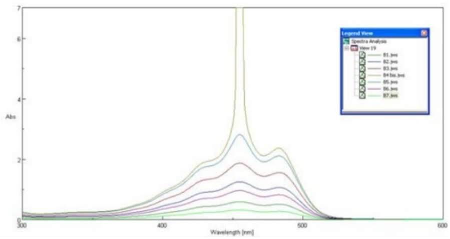

Figure 3. Linearity range of β-carotene in DMSO

Precision

The method repeatability results are presented in Table 3 and Figure 4. We assume that the within-samples standard deviation is the same throughout the range, in order to estimate it we evaluate both, in between the samples (intra-individual variability) and in between the analyst's repeated measurements (inter-observer variability), the maximum RSD, often regarded as the closeness of agreement was 1.88%; Karnjanawipagul P., and W. Nittayanuntawech, 2010).

Table 3. β-carotene repeatability and reproducibility

Sample	Analyst 1			Analyst 2		
1	0.1278	0.1282	0.1286	0.1284	0.1280	0.1278
2	0.1264	0.1262	0.1267	0.1265	0.1262	0.1273
3	0.1279	0.1274	0.1274	0.1272	0.1268	0.1281
Mean	0.1274	0.1273	0.1276	0.1274	0.1270	0.1277
SD	0.0008	0.0010	0.0010	0.0010	0.0009	0.0004
RSD	0.6585	0.7910	0.7533	0.7544	0.7217	0.3164

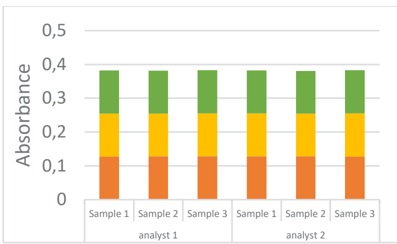

Figure 4. Repeatability and reproducibility of β-carotene in DMSO

CONCLUSIONS

The spectrophotometric detection of β-carotene from red yeasts was successfully developed having the DMSO as an extractive solvent. The current method met both the efficiency and speed tests for three batches of red yeast pigments. Additionally, simultaneous assays were accomplished, having high scores of repeatability and reproducibility. This instrumental method is a cheap, and efficient, suitable for β-carotene determination and retinol and retinoic acid estimation from conventional and nonconventional poultry feed additives such as yeast. Furthermore, the current method presents a great potential for the rapid pigment evaluation and might be suitable for broilers serum blood β-carotene determination.

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EFFECT OF OREGANO ESSENTIAL OIL ON THE PERFORMANCE AND MICROBIOLOGICAL STATUS OF SUCKLING PIGLETS

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Abstract

Essential oils are gaining more and more importance in the animal nutrition, due to their antimicrobial and immunostimulant properties. This experiment aimed to assess the effect of oregano essential oil (OEO) on feed intake growth performance, health and microbiological status of suckling piglets. A total of 71 piglets, originated from 8 sows, were randomly allocated to two treatments - control (C - 35 piglets) and experimental (OEO - 36 piglets). Sows from C group received an antibiotic-free diet, and sows from the OEO group - control diet + 0.500 g/kg feed OEO. Piglets from the OEO group were treated orally by 1 cm^3/day by an emulsion of OEO from day 3 till day 14 of age and by 1 g/kg oregano powder added to creep feed from day 14 till day 35 of age. Application of OEO significantly increased sows' feed intake during the third and fifth weeks of lactation ($P<0.01$). In general, the use of oregano emulsion and powder had no effect on the growth performance and health status of suckling pigs. No significant difference was found in the results of bacteriological tests for the isolation of E. coli and Salmonella spp. of rectal swab samples between the OEO and C groups.

Key words*: ADG, E. coli, growth performance, oregano essential oil, piglets*

INTRODUCTION

Pork production is associated with a large number of antibiotic applications, especially in the pig weaning process and immediately after. However, overuse of veterinary antimicrobials has led to the development of antibiotic resistance, a serious risk for both animals and humans. The growing concern of the public about health made it necessary the European Union to officially ban growth promoters by a directive (Council Directive 70/524/EC - ECR, 1998, entered into force after 1.01.2006). In view of the total ban on the use of antibiotics after 2018, a variety of alternatives are constantly being sought that aim to stabilize the intestinal ecosystem by encouraging the development of beneficial bacteria and reducing the number of pathogenic microflora. Over the past twenty years, a large body of research in monogastric nutrition has been conducted, involving probiotics, prebiotics, acidifiers, plant extracts, nutrients such as copper and zinc, as well as the more unconventional antimicrobial peptides, recombinant enzymes, soil materials, egg protein, rare earth elements, and others. (Thacker, 2013; Pirgozliev et al., 2019a). The success of using such means is still unproven and controversial. For this reason, establishing the concentrations and the combinations of various herbs, herbal extracts, essential oils, probiotics and other substances is the subject of a number of studies both abroad (Bravo et al., 2014; Karadas et al., 2014; Pirgozliev et al., 2015; 2019b) and in our country (Zapryanova and Ignatova, 2020; Ivanova-Peneva et al., 2010; Ivanova-Peneva, 2012; Ivanova et al., 2016; Ivanova et al., 2018; Ivanova and Nikolova, 2021; Ivanova et al., 2022). Special attention is paid to essential oils, which are capable of increasing food intake, stimulating growth and digestion. In addition, they possess antimicrobial, antioxidant and immunomodulating properties (Pirgozliev et al., 2019c; Valdivieso-Ugarte et al., 2019). The herb oregano and its varieties (*Oreganum vulgare*, *Oreganum heracleoticum*) have been used successfully as growth stimulants (Bankova et al., 2019; Neil et al., 2004), and for the control of post-weaning diarrhea in pigs, due to the high antimicrobial activity to *Escherichia coli*, *Salmonella* spp., *Klebsiella pneumoniae* (Stamenic et al., 2014) as well as immunostimulating effect (Camps, 2005; Walter & Bilkei, 2004). Hofmann et al. (2021) also demonstrated that oregano essential oil (OEO) altered the expression of genes related to the adaptive immune response in the small intestine of pigs. This experiment aimed to assess the effect of OEO on growth performance, health and microbiological status of suckling piglets.

MATERIALS AND METHODS

An experiment has been performed at Experimental unit of Agricultural Institute – Shumen. A total of 71 Danube White piglets were involved in the study. The pigs originated from 8 sows, which weaned similar number of piglets. The sows were allocated to two groups following randomization - control (35 piglets) and experimental (36 piglets). The animals were randomized to number of parity, number and weight of piglets in previous farrowing, date of conception. During the pregnancy, the sows were reared in group pens and moved to the farrowing crates a week before the farrowing. The animals were fed two antibiotic-free diets following randomization. A basal diet was produced that contained 12.736 MJ Metabolizable Energy and 18.40 g CP (Table 1). The basal diet was split on two batches as one of them was fed as it is (Control - C) and the other was supplemented with 0.5 g per kilogram diet of OEO (DOSTO©Oregano, Dostofarm, Germany). It is 100% pure natural organically certified OEO of *Origanum vulgare* L., subsp. *hirtum* var. *Vulkan* (DOS 00001), at a concentration of 7.5% (Rychen et al., 2017) and is the only one officially certified by EFSA feed supplement.

All diets were fed in a mash form. Feed intake was determined on a daily basis and animals were fed depending on number of piglets in the litter. Piglets obtained creep feed from day 7 until weaning at day 35 of age. From day 3 to day 14 of age each piglet from the experimental group individually received OEO emulsion at dosage of 1 ml per day. From day 14 to day 35 of age, all piglets received the same creep feed diet supplemented by 1 g OEO per kilogram feed. During the study, the following variables were measured:

- daily feed intake of sows for the entire study period;
- daily creep feed intake of piglets;
- live weight of piglets at birth, on day 7, 14, 21, 28 and at weaning at 35 days of age;
- average daily gain, calculated on weekly basis and for the whole period of the study;
- health condition of piglets. The evaluation of health condition was made on daily basis according to methodology of Camps (2005). The state of faeces and the presence of diarrhea were evaluated from score 1 to 4, as follows: 1 - were normal dry faeces with brown colour; 2 - faeces with increased water content with yellow-brown colour; 3 - watery faeces with yellow colour, 4 - white or yellow diarrhea.

To carry out the microbiological examinations of the faeces of the suckling pigs from the

Table 1. Chemical composition of the experimental diets

Components	**(%)**
Maize	-
Wheat	50.00
Barley	24.17
Soybean meal	23.50
Lysine, 98%	0.08
Calcium carbonate	1.30
Monocalcium phosphate	0.30
Vitamin mineral premix*	0.25
NaCl	0.40
Total	**100.00**
Metabolizable Energy, MJ	12.736
Crude Protein, g	18.4
Lysine, g	0.97
Methionine + Cystine, g	0.65
Treonine, g	0.68
Triptophan, g	0.25
Crude fat, g	1.29
Crude fiber, g	3.94
Ca, g	0.75
P, g	0.60

*Content of vitamin mineral premix: Vitamin A - 4,000,000 IU/kg, Vitamin D3 - 800,000 IU/kg, Vitamin E - 50,000 mg/kg, Vitamin K3 - 2,000 mg/kg, Vitamin B1 - 1,000 mg/kg, Vitamin B2 - 2,000 mg/kg, Niacin - 9,000 mg/kg, Calcium D pantothenate - 7,000 mg/kg, Vitamin B6 - 1,500 mg/kg, Biotin - 100 mg/kg, Folic acid - 650 mg/kg, Vitamin B12 - 15 mg/kg, Iron - 70,000 mg/kg, Copper - 6,000 mg/kg, Zinc - 40,000 mg/kg, Manganese - 20,000 mg/kg, Iodine - 100 mg/kg, Selenium - 160 mg/kg, Antioxidant BHA - 60 mg/kg, Antioxidant BHT - 160 mg/kg, Preservative Lemon acid - 160 mg/kg, Preservative Phosphorus acid - 120 mg/kg control (38 pcs.) and the experimental group (38 pcs.), on day 14 and day 28 days of age, rectal swab samples (RSS) were taken in sterile containers with 0.1% buffered peptone water and 0.85% sodium chloride. The samples (76 pcs.) were examined for the detection of pathogenic *E. coli* and *Salmonella* spp.

For the isolation of *E. coli*, the following media were used: meat peptone broth (MPB) and selective differentiation media to determine the fermentation activity of the isolates - lactose decomposition: brilliant green phenolroth agar, MacConkey agar and Lewin's, Endo's media. For biochemical identification of the isolates, we applied Hi *E. coli* test. To determine the serogroup affiliation of *E. coli*, agglutinating sera were used - O8, O9; O20, O74, O78, O138; O139, O141, O149 and O157. To detect the fimbrial factors, we used Minka agar and slide agglutination with specific sera, respectively F4, F5 and 987P. Blood agar was used to prove hemolyticity of the isolated *E. coli*. The tests for the isolation of *E. coli* were performed according to the Standard of SIV 2789-88, Farm animals, Methods for the laboratory diagnosis of colibacteriosis, Group With 79, in calves and pigs.

To isolate *Salmonella* spp. we used Buffered Peptone Water Granulated; Semisolid Rappaport Vassiliadis Medium Base Modified; Novobiocin supplement; Xylose-Lysine Deoxycholate Agar; Brilliant Green Agar Base Phosphates; Nutrient Agar; Brain Heart Infusion Broth; kit for biochemical identification HiSalmonella Identification kit -KBO11. We developed the samples according to Standard EN/ISO 6579-1:2017.

Data were statistically analyzed by one way ANOVA (Minitab 17). Statistically significant differences were determined at $P < 0.05$.

RESULTS AND DISCUSSIONS

The results of the sow feed intake are shown in Table 2. No statistically significant differences were found in the sow feed intake for the entire lactation. However, as the lactation progressed, in the OEO treatment group in comparison to control, an increase in the feed intake was observed. In the third week, the difference between the two groups was highly significant ($P = 0.007$), as well as in the last week of lactation ($P = 0.001$). This is most likely due to the appetite stimulating properties of oregano essential oil. Our results are in agreement with those obtained by Amrik & Bilkei (2004), in whose study multiparous sows in the experimental group showed higher feed intake compared to the control group. They suggest that the particular aroma of oregano enhances feed palatability, leading to higher *ad libitum* feed intake and weight gain of piglets. Khajarern & Khajarern (2002) reported in sows, oregano essential oils not only act as alternative antibacterial stimulants, digestive aids and appetite enhancers, increasing daily feed intake ($P<0.05$), but when used as natural feed additives, also act as growth enhancers, reproductive ability and milk production.

The results of the piglets' growth performance, average piglet weight and average daily gain (ADG) are shown in Figure 1 and Table 3.

Birth weight was not statistically affected by treatment (3.88% - $P>0.05$), however, there was an increase in piglet body weight in the control group at week one of age compared to the experimental OEO group by 12.23% ($P>0.05$), resulting in statistically significant increase in ADG by 28.57% ($P = 0.020$). This result could be due to the stress of the application of oregano emulsion every day manually to every pig from the OEO group, from the third day of age. The opposite effect in piglet weight development was observed during the second week of age. Repeated measures ANOVA showed a trend for increased ADG in piglets from OEO group by 15.58% ($P = 0.071$).

It is possible that the initial stress of the application of the emulsion is overcome and the effect of the etheric oil as a growth stimulant is manifested. There was no significant effect at the end of the study at weaning of piglets and the ADG was practically equal (0.204 kg/piglet/day in the control and 0.214 kg/piglet/day in the OEO treatment group). It is necessary to notice that the creep feed intake by piglets was very small and it was very difficult to be measured as a big part of it was used as bedding into the piglet compartment.

Table 2. Sow feed intake during lactation per week

Items	Control		Treatment (OEO)		*P*-value
	Mean	SD	Mean	SD	
Sow feed intake week 1 (kg/day)	1.786	0.568	1.929	0.690	0.401
Sow feed intake week 2 (kg/day)	3.250	1.590	3.732	1.450	0.241
Sow feed intake week 3 (kg/day)	5.196^{A}	1.442	5.964^{B}	0.131	0.007
Sow feed intake week 4 (kg/day)	5.625	1.267	6.018	0.095	0.123
Sow feed intake week 5 (kg/day)	6.304^{A}	0.254	6.482^{B}	0.094	0.001
Sow feed average overall (kg/day)	4.468	2.053	4.786	1.849	0.174

Legend: OEO Oregano Essential Oil; SD - Standard Deviation; Superscript letters A-B represent statistical significance at $P < 0.05$

Table 3. Treatment effect on piglet growth performance

Items	Control		Treatment (OEO)		*P*-value
	Mean	SD	Mean	SD	
Average birth live weight (kg)	1.777	0.510	1.708	0.276	0.480
Average live weight 1-7 days (kg)	2.706^{A}	0.849	2.375^{B}	0.430	0.041
Average live weight 7-14 days (kg)	4.074	1.319	3.992	0.830	0.752
Average live weight 14-21 days (kg)	5.477	1.538	5.542	1.066	0.837
Average live weight 21-28 days (kg)	7.200	1.793	7.333	1.599	0.742
Average live weight 28-35 days (kg)	8.931	1.763	9.186	1.570	0.522

Legend: OEO Oregano Essential Oil; SD - Standard Deviation; Superscript letters A-B represent statistical significance at $P < 0.05$

A part of it was also mixed with urine and feces and probably it was not possible to consume the oregano powder added to the creep feed. On the other hand, the intensive smell, due to the high level of essential oil, could presumably reduce the palatability of the diet, leading to poorer intake and growth.

These results are similar to those from a previous study with the addition of oregano to the feed of sows and suckling piglets as a ground oregano herb from the whole plant (Ivanova-Peneva & Kanev, 2014). The results from these two experiments with suckling piglets are different from the results from our earlier study testing the effect of ground dry oregano herb (Ivanova-Peneva et al., 2010). Statistically significant difference in ADG from day 1 to day 40 of age of the piglets in one of the experimental groups was established in comparison to the control (*P*<0.05) and in comparison, to the water solution of oregano herb treatment group (*P*<0.01). Previous studies also did not show consistent results, probably due to the application of a mixture of five herbs including oregano as well (Ivanova-Peneva et al., 2006).

Results from the study made in Wageningen University of testing the effect of DOSTO©Oregano powder on sow and piglet performance added to the sow's feed in the quantity of 750 mg/kg feed reveal the functional mechanism of the essential oil (Auge, 2013). Its basic component carvacrol was found in milk, urine and feces of piglets (58% of dietary intake). The discovery of carvacrol in milk probably shows that the piglets could receive the essential oil directly from the mother's milk. This can improve the health of their gastrointestinal tract due to the antimicrobial effect of oregano essential oil, as well as to a better condition of the sows' udder, expressed in a lower somatic cell counts in milk. In this study, there was an effect on the birth weight of the piglets in the OEO sow group and this effect could be due to a stimulation of the production of more endogenous enzymes and better digestion of the feed. The total weaning weight of the piglets also showed a trend to increase, although an effect on the weight growth performance, expressed as ADG and average weight at weaning was not established, similarly to the results from this study and from other studies with suckling and weaning pigs (Neill et al., 2006; Ariza-Nietto et al., 2011; Hall et al., 2021). Hall et al. (2021) found that the supplement (1% essential oil of *Origanum*

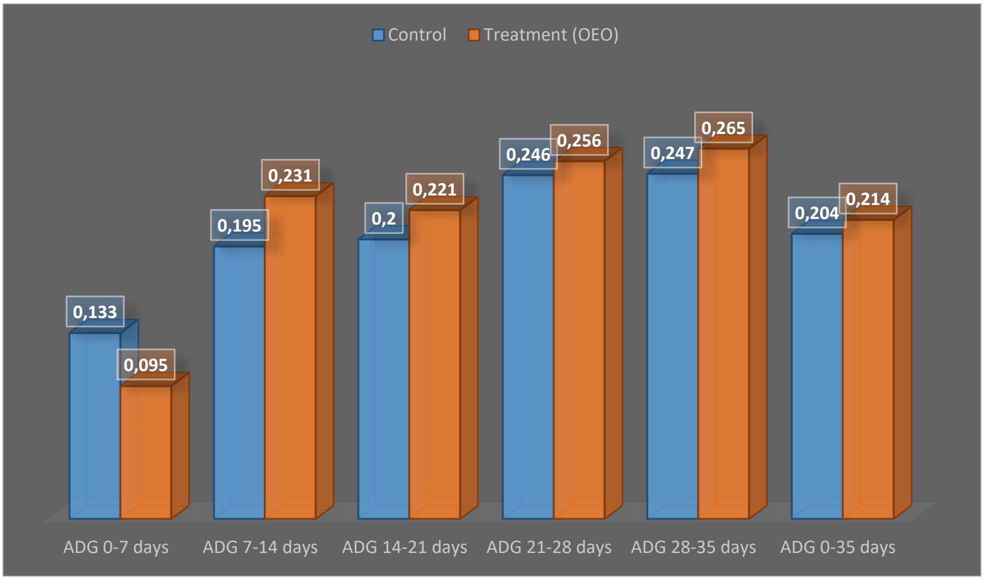

Figure 1. Average daily gain (ADG) of control and treatment (oregano essential oil - OEO) groups, kg/pig/day

vulgare ssp. *hirtum*) had a positive impact on the lifetime performance of pigs from birth to slaughter, with reduced medication use recorded, which may be due to changes in the microbiome early in life through maternal transfer. However, no increased intake of the creep feed was observed and and accordingly no better ADG was established in OEO treated piglets.

The most common reason of diarrhea in suckling, weaning and growing pigs is enterotoxigenic *Escherichia coli* (ETEC). ETEC are characterized by the ability to produce two types of virulence factors: adhesins that facilitate binding to specific enterocyte receptors for intestinal colonization and enterotoxins responsible for secretion of fluid (Dubreil et al., 2016). The best characterized adhesins are expressed in the context of fimbriae, such as F4 (also designated K88), F5 (K99), F6 (987P), F17 and F18 fimbriae. Once established in the small intestine of animals, ETEC produce enterotoxins that cause diarrhea. Table 4 shows the *E. coli* RSS positive samples from the control and experimental (OEO-treated) groups. In the researches of RSS from the piglets of the control group (n = 19), taken on day 14, are isolated 5 samples positive for *E. coli*: 2 samples O8:F5+:Hem-, 2 samples O139:F4+:Hem+ and one with *E. coli* O non-typifying (ONT):F-:Hem+. In the tests of RSS (n=19) from the piglets at day 14 day of age from the experimental group, 4 positive samples were detected, 2 samples RSS with *E. coli* O8:F5+:Hem- and 2 samples RSS with *E. coli* O139:F4+:Hem+. In researches of RSS from piglets at day 28 of age, equivalent results were observed in both groups. In both groups 5 samples positive for *E. coli* were isolated: 2 *E. coli* O8:F5+:Hem-, 2 *E. coli* NT:F-: Hem+, and one *E. coli* O139:F4+:Hem+. Investigating the prevalence of enteritis in pigs in 8 pig farms, Petkova (2017) found that ETES that produce fimbriae (O139:F5) can cause enteritis in both suckling and weaned pigs, which is according to the evidence in this research of hemolytic *E. coli* O139:F4+ from RSS of suckling pigs.

Table 4. Results from investigation of rectal swab samples (RSS) from suckling piglets, raised with and without oregano essential oil (OEO)

Piglets' age	Control group (n = 38)				OEO group (n = 38)			
	E. coli	**O-sero-group**	**Fimbrial factor**	**Hemo-lysis**	***E. coli***	**O-sero group**	**Fimbrial factor**	**Hemo-lysis**
14 days	Pos.	ONT	-	+	Pos.	O8	F5	-
14 days	Pos.	O8	F5	-	Pos.	O8	F5	-
14 days	Pos.	O8	F5	-	Pos.	O139	F4	+
14 days	Pos.	O139	F4	+	Pos.	O139	F4	+
14 days	Pos.	O139	F4	+	-	-	-	-
Total	5	5	4	3	4	4	4	2
14 days	Pos.	ONT	-	+	Pos.	ONT	-	+
28 days	Pos.	ONT	-	+	Pos.	ONT	-	+
28 days	Pos.	O8	F5	-	Pos.	O8	F5	-
28 days	Pos.	O8	F5	-	Pos.	O8	F5	-
28 days	Pos.	O139	F4	+	Pos.	O139	F4	+
Total	5	5	3	3	5	5	3	3

No significant differences between the results in the control and experimental groups can be seen (Table 4). Out of 19 examined RSS from pigs at day 14 of age from the control group, 26.3% were positive for *E. coli*, similar to the OEO group, in which 21.05% were positive. *E. coli* of serogroup O8, O139 and O non-typing were isolated from 4 RSS in the experimental group and from 5 RSS from the control group in the first examination. Equal numbers positive samples (5 RSS) in both groups were found in the second examination at day 28 of age. *Salmonella* spp. was not isolated from neither of the 76 examined RSS taken on day 14 and day 28. During the clinical observation of pigs from both groups, diarrhea was recorded for one day in one piglet from the control group, from which *E. coli* O139:F4:Hem+ was isolated on the day 14. The lack of obvious clinical signs in piglets from which pathogenic *E. coli* was isolated could be explained by the fact that both sows and suckling pigs were treated with oregano in a different form. However, we need more trials with larger number of piglets to be able to prove such a causal relationship. For the entire study period, the condition of the piglets was rated as 1 - normal, well-formed, firm brown stools, in contrast to the previous trials conducted with suckling pigs (Ivanova et al., 2010; Ivanova & Kanev, 2014; Ivanova et al., 2016). This could be due to the well-cleaned and disinfected farrowing room of the sows and the arrival of the piglets in a stable microbiological environment. Information on the growing conditions should be taken into account for a more complete interpretation of the experimental data. Simitzis et al. (2010) pointed out that well-nourished and healthy animals do not respond to growth-promoting additives, for example OEO. In the same context, Gāliņa & Valdovska (2017) concluded that oregano essential oil exerts its effects only during pathological conditions. Pirgozliev et al. (2014) also found that the beneficial effects of essential oils were more pronounced in less hygienic housing conditions. In contrast to Hall et al. (2021), who demonstrated a relative reduction of *Enterobacteriaceae* in feces from pigs at day 14 and 28 of age originating from sows receiving OEO, in the present experiment we found no significant differences in the carriage of pathogenic *E. coli* in the control and experimental piglets groups. In a study of the effect of combining benzoic acid with an essential oil, Rodrigues et al. (2020) found no difference in indicators of diarrhea and microbial population, in the experimental and control groups of piglets over 21 days of age, but reported an improvement in the growth indicators of the piglets. We share the opinion of Canibe et al. (2022) that essential oils show potential to reduce the development of enteritis in piglets and there seems to be no doubt that some of them have essential benefits, especially their specific antimicrobial effect against ETEC. However, according to them, a major challenge is the mixing of essential oils with organic acids, particularly benzoic acid, which complicates the assessment of the effects of essential oils on their own. Another major challenge in general evaluation of the results and providing recommendations is that none of the experiments are directly comparable because they vary widely in the type, amount, purity, and combinations of essential oils involved. The results of the studies depend on factors related to age, health and immune status of the pigs used in the experiment, the feed fed and the farming technology, the dose and concentration of the added product, the duration of the treatment and the level of stress and hygiene, as well as research design.

CONCLUSIONS

The application of OEO emulsion of 1 ml per piglet per day orally and OEO powder of 1 g per kg of feed in suckling piglets paralleled with the application of OEO powder of 0.5 g per kg of feed in sows, showed a trend for an increased suckling piglet growth performance from day 7 to day 14. Application of OEO significantly increased sows' feed intake during the third and fifth weeks of lactation ($P<0.01$). In general, the use of oregano emulsion and powder had no effect on the growth performance and health status of suckling pigs. No significant difference was found in the results of bacteriological tests for the isolation of *E. coli* and *Salmonella* spp. of rectal swab samples between the experimental and control groups.

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THE FEEDING EFFECTS OF URONIC ACID EXTRACTION FROM *Sargassum crassifolium* ON UNSATURATED FATTY ACIDS AND THE IMMUNITY OF LOHMAN CHICKEN EGGS

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Abstract

Antibiotics are currently not allowed to be used because they can make pathogenic bacteria resistant and leave residues in products. The purpose of this study was to determine whether Lohman chicken eggs' immunity was affected by drinking water containing uronic acid extracted from Sargassum crassifolium (S. crassifolium). Sixty laying hens were divided into two groups: 1) chickens fed commercial feed with antibiotics, and 2) chickens fed feed without antibiotics. The chickens were randomly assigned to one of five treatments that included brown seaweed in the drinking water, A1 = 0.0% (control); A2 = 2.5%; A3 = 5.0%; A4 = 7.5%; A5 = 10.0%. Five treatments, two factors, and three replications were used in the completely randomized study design. Six laying hen heads were included in each replication. Titer antibody and unsaturated fatty acid were different between treatments, but Salmonella sp. infection was the same. It came to the conclusion that the lohman chicken eggs' immunity and unsaturated fatty acid levels were both enhanced by the uronic acid extracted from S. crassifolium.

Key words: *fatty acid, immunity, Lohman chicken, Sargassum crassifolium, uronic acid.*

INTRODUCTION

Sargassum crassifolium (*S. crassifolium*) is a member of the Phaeophyceae (brown algae) family. It has true roots, stems, and leaves (Yenusi et al., 2014), a variety of forms, and a predominant brown or blonde color that does not change with drying (Merdekawati & Susanto, 2009). It has been demonstrated that brown seaweed (*S. crassifolium*), which has major components such as sugar, sulfate, and uronic acid, acts as an antiviral and antibacterial agent (Mandal et al., 2007). Carotenoids and polysaccharides are found in *S. crassifolium*. Polysaccharides aid in digestion, reduce blood lipid and cholesterol levels, and possess anti-thrombotic, anticancer, antioxidant, antiproliferative, anti-inflammatory, anticoagulant, and antiproliferative properties (Zhao et al., 2005). Polysaccharides, dietary fiber, minerals, proteins, amino acids, vitamins, polyphenols, and carotenoids (Burtin, 2007) are among the antioxidants found in seaweed, according to numerous studies.

Because it hasn't been used to its full potential, seaweed can be processed into ingredients for animal feed. According to March et al. (2013) and Anggadiredja et al. (1996), seaweed is a natural source of non-starch polysaccharides that contains a lot of crude fibers. Its bioactive factors affect the digestive process, changing the microflora in the caecum and allowing laying hens to use nutrients effectively.

If you want to eat eggs, you should look at their quality. The search for various forms of unsaturated fatty acids, which the body needs to prevent a variety of diseases, began. Customers tend to prefer eggs of higher quality. Also, very important for getting good egg parts is feeding, especially feed with a lot of nutrients in it (Kereh et al., 2019). However, livestock can be harmed by microbes found in food, water, or the air, such as viruses or bacteria. Bacteria, such as *Salmonella* species group, frequently transmit contamination of chickens to consumers through the hatching, growth, and post-harvest stages (Gantois, 2009). These bacteria will have an impact not only on the health of livestock but also on the safety of meat or egg products that humans will consume. This has been overcome through vaccination, sanitation, and antibiotic use, among other methods.

This effort is useful, but it has some limitations, like some bacteria strains that are resistant to antibiotics (Devegowda et al., 1997).

Antibiotics are used to stop pathogens from growing (Farhad & Farida, 2011; Teteh et al., 2016). However, the use of antibiotics in feed has been restricted due to their tendency to increase pathogen bacteria's resistance (Abdulhasan, 2018; Santoso et al., 2018). As a result, antibiotic alternatives made from natural ingredients are required in feed formulas (Abaza, 2007; Winsisch et al., 2008; Abbas, 2013; Aqil, 2016; Mahfuz et al., 2017; Voemesse et al., 2018) in order to produce meat and egg products that are safe, healthy, and competitive (Mattjik & Sumertajaya, 2002; Rusli et al., 2015).

Utilizing *S. crassifolium* containing uronic acid may become an alternative to antibiotics. *S. crassifolium* has not yet been reported as a potential seaweed additive for feed ingredients, particularly feed additives. Therefore, the purpose of this study was to investigate how Lohman chicken eggs' immunity was affected by uronic acid extracted from *S. crassifolium* as a substitute for antibiotics.

MATERIALS AND METHODS

Research Material

This study used 120 Lohman strains aged 22 weeks and brown seaweed (*S. crassifolium*). Antibiotic-containing feed and antibiotic-free feed were the commercial feeds used in the study. Brown seaweed (*S. crassifolium*) extract was added to drinking water at concentrations of 0, 2.5, 5.0, 7.5, and 10%.

Table 1 displays the feed's nutritional content. A 35 x 36 x 42 cm individual battery cage with a feeding area, drinking water, and lights (16L/8D lighting system) was used.

Before beginning treatment, the chickens were introduced to the provisional feed and water for a week. For three months, the chicken needed to be looked after.

Preparation of Seaweed extract

100 grams of dried seaweed were mixed with ethanol (90 percent) (5:1), stirred for three hours, left to stand for 24 hours at room temperature, and then concentrated at 50 degrees Celsius to produce seaweed extract.

Table 1. Nutrient content of feed

	Nutrient composition
Dry mater (%)	93.02
Ash (%)	10.77
Crude protein (%)	18.12
Ether extract (%)	5.63
Crude fibre (%)	6.16
BETN (%)	52.34
Gross energy (kcal/kg)	37.34
Calcium (%)	5.85
Phosphor (%)	0.71

Feeding Trial

Twenty-two 18-week-old Lohman strains were divided into two groups: 1) chickens fed antibiotic-laden commercial feed and 2) chickens fed antibiotic-free feed One of the five treatments containing brown seaweed was randomly assigned to the chickens: 0, 2.5, 5.0, 7.5, or 10%) in the water used for drinking. In the morning (at 7 a.m.) and afternoon (at 17 p.m.), ad libitum feed and water were given to the animals.

Variables observed

The variables observed in this study included the following: egg unsaturated fatty acid, the immunity of laying hens to Salmonella sp. Detected by coagglutination test and antibody titer detected by serological tests

Trial Design and Data Analysis

An experiment with a completely random design and three replications was carried out in a 5x2 factorial arrangement. Six laying hens lived in each replication. The first factor was the amount of brown seaweed (*S. crassifolium*) present in the water used for drinking (A1 = 0% *S. crassifolium* in the control); 2.5% *S. crassifolium* in A2; A3 contains 5% *S. crassifolium*; 7.5% *S. crassifolium* in A4; A5 is *S. crassifolium* 10.0%. The presence or absence of antibiotics in the feed was the second factor (B1 indicates feed with additional antibiotics, B2 indicates feed without additional antibiotics). The data were analyzed using analysis of variance followed by Duncan's multiple range test and the orthogonal polynomial test using the SPSS® 21.0 statistical software program.

RESULTS AND DISCUSSIONS

Coagglutination and serological tests revealed that Lohmann chickens' immunity was affected by the administration of uronic acid extracted from *S. crassifolium* in drinking water as an alternative to antibiotics. The chicken's resistance to Salmonella sp. detected by a test of coagglutination. All of the research chickens treated with the uronic acid level of *S. crassifolium* with or without antibiotics in their feed had positive reactions to Salmonella sp., according to the analysis of chicken blood coagglutination. All chickens treated with *S. crassifolium* uronic acid levels exhibited immunity against *Salmonella* sp., as demonstrated by these results.

Serological tests revealed the contained antibody titer of the poultry blood serum. According to the findings of the statistical analysis performed on the antibody titer of Lohman chickens, the treatment of feed without antibiotics with a level of 10% *S. crassifolium* (B2A5) yielded the highest antibody titer in comparison to other treatments at the end of the study (34th week), whereas the treatment of uronic acid levels had non-significant differences (P>0.05) at the beginning of the study (3rd week) (Table 2).

Table 2. Effect of uronic acid level on chicken antibody titer

Factor	3th week			34th week		
	B1	B2	Average	B1	B2	Average
A1	2.33	0.33	1.33	2.33a	2.33a	2.33
A2	2.67	2.00	2.33	3.67b	2.33a	3.00
A3	3.00	1.67	2.33	2.67a	3.00a	2.84
A4	2.67	2.67	2.67	3.33a	3.00a	3.17
A5	3.00	3.33	3.17	3.00a	4.67b	3.84
Average	2.73	2.00		3.00	3.07	

A1 = 0% Uronic acid (control), A2 = 2.5% Uronic acid, A3 = 5% Uronic acid, A4 = 7.5% Uronic acid, A5 = 10% Uronic acid, B1 = Feed with antibiotic; B2 = Feed without antibiotic. A different superscript in the same row shows a significant difference (P<0.05).

The effect of the treatment of brown seaweed extract (*Sargassum crassifolium*) in the drinking water of laying hens on egg palmitic acid levels can be seen in Table 3. *Sargassum crassifolium* 10% were significant (P<0.01) have higher unsaturated fatty acid than level of *Sargassum crassifolium* 0% and 2.5%, but have non significantly (P>0.05) than level of *Sargassum crassifolium* 7.5%.

With a level of uronic acid extracted from *S. crassifolium* present in drinking water, feed intake (g/head/d) tended to be higher without antibiotics than with antibiotics. This shows that *S. crassifolium* - derived uronic acid can increase feed intake and facilitate feed digestion in drinking water.

Table 3. Effect of uronic acid level on egg fatty acid

Factor	palmitic		oleic		linoleic	
	B1	B2	B1	B2	B1	B2
A1	19.20	19.28	33.41	33.35	7.67	7.40
A2	20.37	20.78	36.68	36.18	10.27	10.81
A3	20.66	22.18	37.80	39.71	10.28	11.14
A4	23.27	22.68	38.07	38.69	10.50	9.62
A5	21.52	22.04	38.98	38.38	10.03	10.43
Average	21.00	21.39	36.99	37.26	9.75	9.88

A1 = 0% Uronic acid (control), A2 = 2.5% Uronic acid, A3 = 5% Uronic acid, A4 = 7.5% Uronic acid, A5 = 10% Uronic acid, B1 = Feed with antibiotic; B2 = Feed without antibiotic. A different superscript in the same row shows a significant difference (P<0.05).

Zhao et al. (2005) mentioned that *S. crassifolium* might make it easier to digest food. According to the findings of this study, the alginate that is derived from the uronic acid that is extracted from *S. crassifolium* probably played a significant role in increasing the feed intake of Lohman chickens. According to Brownlee et al. (2005), alginate is a soluble fiber that reduces blood glucose levels, reduces intestinal lumen toxicity, eliminates harmful microbial colonies, absorbs toxins in the colon, and alters intestinal microflora. Because of these conditions, more feed is taken in and the rate at which the digestive tract is emptying faster.

Eggs have a low percentage of unsaturated fatty acids due to the transfer of those fatty acids. The linoleic content of egg yolks will rise when fed a source of polyunsaturated fatty acids (PUFA), whereas the oleic content will decrease. The same effect can be seen when uronic acid extracted from *S. crassifolium* is added to drinking water.

Salmonella sp. immunity is present in all chickens treated with *S. crassifolium* uronic acid levels. This is possible due to the immune-modulatory properties of brown seaweed polysaccharides. Both specifically and non-specifically, the immune system's defenses are strengthened by immunomodulators, which also induce non-specific cellular and humoral defense mechanisms. These non-starch polysaccharides arrive in the intestines intact and serve as immunostimulants (Ale et al., 2011) because they are resistant to saliva's digestion and hydrolysis in the mouth, stomach, and small intestine.

At the conclusion of this study, the antibody titer value generally increased when the uronic acid level of *S. crassifolium* in laying hens was higher (Table 1). This demonstrates that layer hens' antibody can be increased with the uronic acid from *S. crassifolium*. Antibodies may be produced by the *S. crassifolium* uronic acid, which may prevent viral replication. According to Han & Marasco (2011), the body's defense against viruses relies heavily on antibody-mediated immune responses. By binding to viral proteins, antibodies prevent viral replication, thereby preventing the replication process.

CONCLUSIONS

Unsaturated fatty acid levels and immunity in Lohman chicken eggs have been raised by administering uronic acid extracted from *S. crassifolium* in water as an alternative to antibiotics.

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ARE HABITATS WITH *Campanula romanica* Săvul. PREFERRED BY THE SOIL FAUNA?

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Abstract

In 2021, from the first time in Romania, soil fauna taxa were investigated from two types of habitats: with and without Campanula romanica, from Măcin Mountains National Park. Some population parameters were analysed as: taxa diversity, numerical abundance, dominance, evenness, equitability, Shannon-Wiener index of diversity. In total, 24 soil fauna groups were identified, with 399 individuals, recording characteristic structure and taxa for each type of plots. Even if the number of identified taxa is almost similar between plots, the numerical abundance was higher in areas without Campanula romanica. In the same time sixteen environmental variables were quantified, as: the thickness of three soil layers, air temperature and humidity, soil temperature and moisture, soil pH, soil penetration resistance, amount of organic carbon, total nitrogen, C/Nt ratio, humus content, potassium content, phosphorous content and the vegetation cover. They had a significant influence on structure composition of the edaphic taxonomic groups from the two types of habitats.

Key words: *Campanula romanica, environmental parameters, soil taxa.*

INTRODUCTION

Due to the ecological requirements, soil fauna could constitute a valuable bioindicators of the ecosystems, many times being possible to evaluate their conservation status through these invertebrates. Some methods for this evaluation are: to identify the taxonomical spectrum of soil fauna, to characterize the environment biotic and abiotic parameters of ecosystems where these edaphic invertebrates live and to demonstrate how these factors influence the structure and dynamics of soil fauna (Koehler & Melecis, 2010; Coleman & Wall, 2015). Many studies in all over the world demonstrated that the vegetation type influence the structure of soil fauna or vice-versa (Zhao et al., 2011; Ulrich et al., 2020; Chiriac & Murariu, 2021). The loss of biodiversity and indirectly of soil fauna would deteriorate the ecosystems functions (Iordache & Neagoe, 2023). The invertebrate decline may contribute to relations between plants and animals, with potential negative consequences for ecosystem services like food provision and soil production (Koehler & Melecis, 2010; Zhao et al., 2011; Ulrich, 2020).

Campanula romanica Săvul. (*C. rotundifolia* L. subsp. *romanica* (Săvul.) Hayek) is a perennial species, hemicryptophyte, with heights between 18 and 30 cm and blooms from June to August. It is a symbol species for Măcin Mountains National Park, endemics for Dobrogea, being identified in Ponto-sarmatic steppes (62C0*) Natura 2000 habitat, having conservative value, according to European Directive Habitats 92/43/EEC (Mihăilescu et al., 2015). It is xerophilous, saxicolous species, which grows in the cracks of the calcareous or granitic rocks, in dry and sunny habitats (Ciocârlan, 2009; Dihoru & Negrean, 2009). In order to establish its actual conservation status, in 2021, a monitoring programme was developed in its habitats from Măcin Mountains National Park. Taking this aspect into account, we consider that the study of soil fauna taxa in monitoring plots with or without *Campanula romanica* will constitute a new and innovative approach of soil invertebrates' ecology. It is for the first time in Romania when such study was accomplished. The main objectives of the present study were: I) to highlight the structural differences of the soil fauna taxa from plots with and without *Campanula romanica*, II) to characterize the environmental parameters from investigated plots, and III) the show the different influence of the abiotic parameters on soil fauna taxa.

MATERIALS AND METHODS

Study area

The research was conducted in July 2021, in Măcin Mountains National Park. It is located in the South-East of Romania, in Dobrogea region, Tulcea county (45º8´49" N and 28º19´51"E). It has an area of 11151.82 hectares, being the most arid mountains from Romania. The climate is continental, with sub-Mediterranean influences in higher areas and with steppic characteristics in the south. Average annual temperatures are 10-11°C and average precipitation is 500 mm, which are extreme values within Romania (Manu et al., 2016). The study was developed in Ponto-sarmatic steppes (62C0*), a favourable Natura 2000 habitat for *Campanula romanica* (CR) (Figure 1). A detailed description of investigated plots is presented in Table 1.

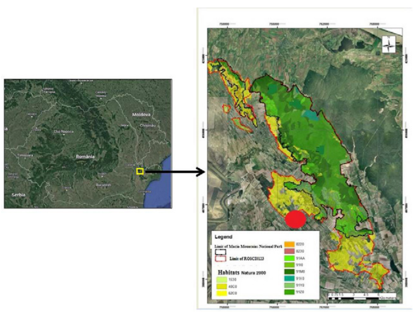

Figure 1. Geographical characterisation of the investigated areas in Măcin Mountains National Park-Romania, in 2021 (red bullet = geographical position of investigated plots with and without *Campanula romanica*)

Soil fauna

In July 2021, twenty two soil samples were collected, using a MacFadyen soil core (5 cm diameter) to 10 cm depth. Due to the fact that the study was made in a Natura 2000 protected area, the number of soil samples was limited. The samples were collected taking into account the presence or absence of *Campanula romanica* (CR) species (eleven samples in plots with *Campanula romanica* and eleven samples in plots without this plant species). The soil fauna groups were extracted using the Tullgren-Berlese method (by natural drying for 20 days) (Macfadyen 1953, 1961; Koehler & Melecis, 2010). Identification of soil fauna groups was performed on the Carl Zeiss stereomicroscope, and their preservation was made in ethylic alcohol of 90^0. The identification of taxa was made using the published identification keys (Brussaard et al., 1997; Ceuca, 2010; Coleman & Wall, 2015; Dindal, 1990; Gîndei & Popescu, 2009; Krantz & Walter, 2009; Orgiazzi et al., 2016; Platnick, 2018).

Environmental variables

In total, sixteen environmental variables were quantified. Within the soil the following parameters were quantified: the thickness of the litter fermentation layer- TOLF (cm); the thickness of humus layer- TOH (cm); the thickness of soil layer- TOS (cm); the temperature -T_{soil} (^{0}C); acidity-pH; moisture content - H_{soil} (%); penetration resistance- RP (PSI), humus (%), organic carbon - C_{org} (%), total nitrogen - Nt (%), carbon: nitrogen ratio - C/N; the content of phosphorus - P_{AL}(mg/kg); content of potassium - K_{AL}(mg/kg). On 5 cm above the soil level the temperature - T_{air} and air moisture content - H_{air} were measured. In total, 20 soil samples were analysed in order to measure these abiotic factors. The thickness of the litter-fermentation layer (OLF), of the humus layer (OH) and of the soil layer (OS) was measured with a graduated ruler (in centimetres), taking into account the morphological properties of the soil sample (colour, texture, consistency) (Chiriţă, 1974). A digital thermo-hygrometer PCE-310 was used to measure air and soil moisture and temperature. Penetration resistance was determined with a soil penetrometer, Step System GmbH, 41010. The pH was measured with a C532 Jasco Consort pH-meter. Quantified chemical analyses were: the amount of organic carbon (humus: wet oxidation; STAS 7184/21-82; PTL 12); total N (Kjeldahl method; STAS 7184/2-85; PTL 09); P_{AL} (extractable phosphorus) was also analysed in ammonium acetate-lactate; STAS 7184/19-82; PTL 19P); K_{AL} (potassium extractable in ammonium acetate-lactate; STAS 7184/18-80; PTL 22). The vegetation cover was determined using pratological method, which take into consideration the percentage participation in biomass of botanical components by economic groups (as grasses, legumes, mosses and lichens, wood species). It is one of the most recommended fast method for determining grassland vegetation coverage (Ivan & Doniţa, 1975; Onete et al., 2021). The average values of environmental variables are presented in Table 2.

Table 1. A detailed description of investigated plots, from Măcin Mountains National Park, 2021

Average values of parameters	With CR	Without CR
GIS coordinates	From N 45^0 03'28.9"; E 28^{0}10'46.7" To: N 45^0 15'19.8"; E 28^{0}21'21.1"	From N 45^0 02'48.7"; E 28^{0}10'43.4" To: N 45^0 15'28.1"; E 28^{0}21'17.9"
Altitude	155 m	176 m
Exposure	50% West; 20% South, 20% North, 10% East	20% West; 40% South, 40% East
Slope	21.10	13.50
Habitat	80% rocky habitat; 20% pastures	20% rocky habitat; 80% pastures
Type of soil	Sandy	Sandy
Total vegetation cover	52.3%	59.55%
Herbaceous layer cover	47.8%	65.91%
Shrub layer cover	2.64%	0.36%
Tree layer cover	0.91%	0
Plant species dominant	*Trifolium pratense* L., *Campanula romanica* Săvul., *Festuca* sp., *Rumex* sp., *Achillea quartata* L., moss, *Ailanthus altissima* (Mill.) Swingle, *Moeringia* sp., Dianthus sp., *Thymus* sp., *Centaurea* sp.	*Thymus zigioides* Griseb., *Teucrium polium* L., *Bromus* sp., *Trifolium pratense* L., *Achillea quartata* L., *Scleranthus* sp., moss, *Trifolium fragiverum* L., *Achillea millefolium* L.
Type of anthropic impact	Grazing, invasive species, tourism	Grazing
Intensity of anthropic impact	Medium	Medium

Table 2. The mean values of investigated environmental variables in plots with and without *Campanula romanica* (CR), from Măcin Mountains National Park, 2021 (± standard error)

Environmental parameters	TOLF	TOH	TOS	Tair
With CR	0.89 ± 0.192	0.32 ± 0.139	3.55 ± 0.207	35.77 ± 0.706
Without CR	0.62 ± 0.202	0.2 ± 0.2	4.4 ± 0.266	36.22 ± 0.921
p	0.387	0.873	0.046	0.948
Environmental parameters	H air	T soil	H soil	RP
With CR	56.97 ± 2.856	26.32 ± 0.699	48.55 ± 3.490	126.37 ± 10.640
Without CR	59.72 ± 2.539	27.25 ± 0.846	58.6 ± 4.023	164 ± 13.182
p	0.325	0.601	0.041	0.022
Environmental parameters	VegCov	pH	Humus	Corg
With CR	65.45 ± 8.981	4.81 ± 0.157	12.74 ± 1.423	7.39 ± 0.826
Without CR	58 ± 11.333	5.54 ± 0.152	8.88 ± 1.145	5.15 ± 0.664
p	0.696	0.004	0.044	0.044
Environmental parameters	Nt	C/N	PAL	KAL
With CR	0.67 ± 0.077	12.91 ± 0.414	26.11 ± 5.439	156.91 ± 26.348
Without CR	0.46 ± 0.053	12.8 ± 0.359	7.24 ± 1.303	187.2 ± 14.369
p	0.043	0.735	0.002	0.384

Data analysis

The population parameters used in the statistical analysis were: the number of taxa, the numerical abundance (number of individuals), dominance (D%), species diversity (Shannon-Wiener index), eevenness (e^H/S) and equitability (J index). For the environmental parameters, the mean values were evaluated, including the standard error (± SE).

The correspondence analysis (CA) was used as an ordination method between biological components. The relationship between the environmental parameters and the number of species was established using canonical correspondence analysis (CCA). The used software also includes standard statistical tests for univariate data, such as the ANOVA test. This analysis of variance is a statistical procedure for testing the null hypothesis, for several univariate samples that are taken from within mite communities that have the same average. The samples are assumed to have a normal distribution and a similar variance (p = is the probability of obtaining a result at least as extreme as the one actually observed, given that the null hypothesis is true). The statistical software package PAST was used (Hammer et al., 2001).

RESULTS AND DISCUSSIONS

Considering the environmental parameters, the plots with *Campanula romanica* were characterized by the highest values of the thickness of litter-fermentation and humus layers, of air humidity, the highest percent of vegetation cover, the highest content of humus, organic carbon, total nitrogen and phosphorous in soil, as well as the C/N ratio (Table 2). Plots without *Campanula romanica*, were characterized by the highest values of the soil thickness, air and soil temperature, soil moisture content, soil resistance at penetration, pH and content of potassium from soil. Making a comparison between the two types of investigated plots, significant differences were obtained between thickness soil layer, soil moisture content, soil resistance at penetration, pH, content of humus, organic carbon, total nitrogen and phosphorous from soil ($p<0.005$) (Table 2). A precious indicator of soil quality is the C/Nt ratio, which indicates of the mineralization capacity of nitrogen. According to soil specialists, a ratio lower than 15 (C/Nt < 15) indicates a high rate of decomposition of organic matter and organic nitrogen, (Chiriţă, 1974; Klarner et al., 2013). In both type of plots, the C/Nt ratio is lower than 15, which indicate a proper rate of organic matter decomposition and a favorable habitat for soil taxa. The presence of organic matter (as higher content of C_{org} or humus) is a favorable factor for development of edaphic invertebrate taxa (as Nematoda, Collembola, Enchytraeidae, Oribatida, etc.), which in turn represent the food source for predator groups (such as Gamasina, Trombidiidae) (Krantz & Walter, 2009; Klarner et al., 2013).

Analyzing the taxonomical spectrum of identified faun groups, we obtained twenty four soil taxa, belonging to two phyla Annelida and Arthropoda (Table 3). Considering the number of soil taxa, the differences between two types of plots is insignificant, the same tendency being observed at characteristic taxa for each investigated areas, even if the Shannon diversity index is slightly increased in plots with *Campanula romanica* (Table 3). If we put into discussion the number of individuals, in plots without *Campanula romanica* (204 individuals), this parameter recorded higher value than that from plots with *Campanula romanica* (195 individuals). The total numerical abundance was by 399 individuals. The dominant soil taxa in both types of plots were Collembola (with a total numerical abundance by 172 individuals), Oribatida (63 individuals) and Chamobatidae (64 individuals). Higher dominance index was obtained in plots without *Campanula romanica*, which demonstrated the increased values of numerical abundance of few taxa, phenomenon highlighted by the decreased values of evenness and equitability indices (Table 3).

Table 3. The number of indiviuals of each identified taxa in studied plots,
with and without *Campanula romanica* (CR), from Măcin Mountains National Park, 2021

Taxonomical classification	Investigated taxa	With CR	Without CR
Phylum Annelida			
Class Clitellata			
Subclass Oligochaeta			
Order Haplotaxida			
Family Lumbricidae	Lumbricidae	1 ± 0.301	1 ± 0.301
Family Enchytreidae	Enchytraeidae	6 ± 1.35	2 ± 0.603
Phylum Arthropoda			
Subphylum Myriapoda			
Class Diplopoda	Diplopoda	2 ± 0.603	
Class Chilopoda	Chilopoda	4 ± 0.674	3 ± 0.646
Subphylum Hexapoda			
Class Entognatha			
Order Collembola	Collembola	56 ± 5.430	116 ± 13.094
Order Diplura	Diplura	1 ± 0.404	
Order Protura	Protura	2 ± 0.301	
Class Insecta			
Order Coleoptera			
Family Curculionidae	Curculionidae	1 ± 0.301	
Order Psocoptera	Psocoptera	4 ± 0.674	3 ± 0.646
Insect larva	Insect larva	7 ± 0.809	8 ± 0.786
Subphylum Chelicerata			
Class Arahnida			
Order Araneae	Araneae		1 ± 0.301
Supraorder Acariformes			
Order Trombidiformes			
Suborder Prostigmata			
Family Trombidiidae	Trombidiidae	1 ± 0.301	
Family Cunaxidae	Cunaxidae		2 ± 0.603
Order Ixodida			
Superfamily Ixodoidea			
Family Ixodidae	Ixodidae		1 ± 0.301
Order Sarcoptiformes			
Suborder Oribatida	Oribatida	41 ± 5.728	22 ± 2.756
Family Galumnidae	Galumnidae		1 ± 0.301
Family Camissidae			
Family Ceratozetiidae	Ceratozetiidae		1 ± 0.301
Family Chamobatidae	Chamobatidae	37 ± 7.579	27 ± 6.846
Family Oribatellidae	Oribatellidae	7 ± 1.120	5 ± 0.687
Suborder Astigmata			
Family Acaridae	Acaridae	1 ± 0.301	
Family Glycyphagidae	Glycyphagidae		1 ± 0.301
Order Mesostigmata			

Suborder Gamasina	Gamasina	6 ± 0.934	7 ± 0.924
Suborder Uropodina	Uropodina	1 ± 0.301	
Acari immatures	Acari-immatures	17 ± 4.803	3 ± 0.904
Total number of taxa		18	17
Total number of individuals		195 ± 14.900	204 ± 23.870
Number of characteristic taxa		7	6
Dominance_D		0.18	0.36
Shannon_H		2.08	1.60
Evenness_e^H/S		0.45	0.29
Equitability_J		0.72	0.56

In plots with *Campanula romanica* some characteristic soil fauna taxa were identified, as: Diplura, Protura, Diplopoda, Trombidiidae, Acaridae, Curculionidae (Figure 2). Diplura is considered the sister group to insects and thus the closest group among the three basal hexapods that include Collembola and Protura. All inhabit soil and subsurface of terrestrial habitats. These invertebrates, due to their soft body and thin cuticle, depend on high humidity and moderate temperatures (Sendra et al., 2021). In soils, diplurans occupy all soil horizons. Larger species occupy the upper layers represented by O and A-horizons, smaller species live in the narrower pores of the B-horizon, where only diplurans with a tiny body (less than 2 mm body length and short appendages) can move (Sendra et al., 2021). Many Acaridae species are associated with bark beetles, even with Curculuionidae, which was identified in plots with *Campanula romanica* (Klimov & Khaustov, 2018).

In plots without *Campanula romanica*, were identified another characteristic taxa: Cunaxidae, Ixodidae, Glycyphagidae and Araneae (Figure 2). Mites from Cunaxidae family are present in various types of ecosystems (forest, grasslands, agricultural fields). They are opportunistic predators, feeding with nematodes, collembolans, phytophagous mites or thrips (Skvarla et al., 2014). Glycyphagidae mites generally are associated with the nests of rodents and insectivores (Krantz & Walter, 2009). Araneae taxon was identified in plots where 80% from investigated habitats were grasslands, with higher soil and air temperature.

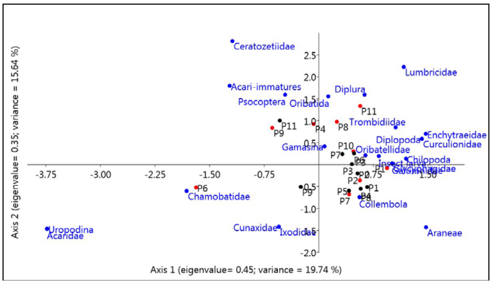

Figure 2. Correspondence analysis (CA) between identified soil fauna taxa and analysed plots (red dots = plots with *Campanula romanica*; black dots = plots without *Campanula romanica*) from Măcin Mountains National Park, 2021

Considering the influence of environmental parameters on the soil fauna taxa, we observed that in plots with *Campanula romanica*, that Gamasina, Chamobatidae were influenced by the thickness of humus layer (TOH), Diplura by the soil content of humus, Oribatida and Oribatellidae by the soil moisture content (Figure 3). In plots without the *Campanula romanica*, taxa as Chamobatidae, Uropodina and Acari-immatures were influence by the Nt and Corg, two abiotic factors which characterize the presence of organic matter in soil. Lumbricidae, Galumnidae and Gamasina were influenced by the soil temperature; Oribatellidae by the soil pH, Araneae by the thickness of soil layer, insect larva by the air temperature and Collembola by the content of potassium from soil (Figure 4).

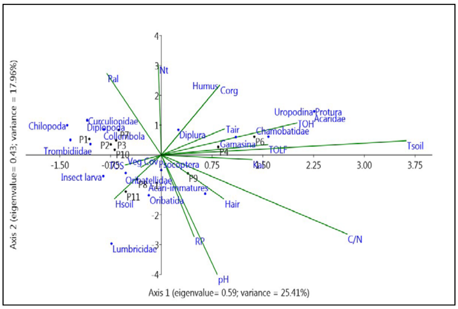

Figure 3. Canonical correspondence analysis (CCA) between identified soil fauna taxa and environmental parameters, in plots with *Campanula romanica*, from Măcin Mountains National Park, 2021

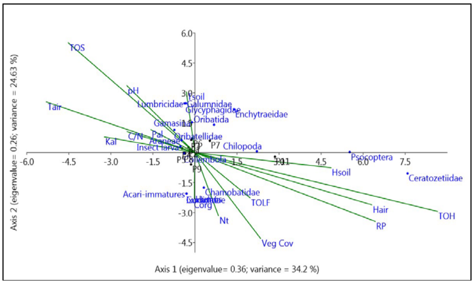

Figure 4. Canonical correspondence analysis (CCA) between identified soil fauna taxa and environmental parameters, in plots without Campanula romanica, from Măcin Mountains National Park, 2021

CONCLUSIONS

In 2021, a study focused on soil fauna taxa was made, in plots with and without *Campanula romanica* from Măcin Mountains National Park. *Campanula romanica* is an endemic species for Dobrogea. It is xerophilous, saxicolous species, which grows in the cracks of the calcareous or granitic rocks, in dry and sunny habitats. These ecological requirements had influenced the composition of soil fauna taxa. We identified 24 soil fauna groups, with 399 individuals, recording characteristic structure and taxa for each type of plots. Even if the number of identified taxa is almost similar between plots, the numerical abundance was higher in areas without *Campanula romanica*. In the same time sixteen environmental parameters were analyzed. They recorded specifically values, characteristic for each type of plots, influencing in various ways the structure of soil fauna. There is a significant interdependence between these environmental variables, influencing the abundance and distribution of edaphic taxonomic groups in studied plots.

As a general conclusion, the microhabitats with *Campanula romanica* are proper for certain soil fauna taxa (as Enchytraeidae, Diplura, Protura, Acari immatures), and for the most abundant groups as Collembola and Acari, but on the other hand the habitats without *Campanula romanica* are preferred by the same dominant soil invertebrates, and by the Cunaxidae and Insect larva, as well.

The present study demonstrated that soil invertebrate groups recorded different structural patterns, in correlation with the type of vegetation and environmental parameters.

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MINERAL OIL HYDROCARBONS (MOH) ANALYSIS IN ANIMAL FEED: A CHARACTERIZATION BASED ON MODERN POLLUTION

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Abstract

This research aims to confirm and quantify the presence of mineral oil hydrocarbons (MOHs) in feed, as well to investigate the contribution of modern pollution sources to the level of contamination. Through simultaneous processes of microwave assisted saponification (MAS), extraction and purification procedures, followed by the LC-GC-FID detection, 8 types of feeds from one of the most polluted areas of the country were analyzed. The results indicated contamination with MOH for most of the feed samples, mineral oil saturated hydrocarbons (MOSH) concentrations above the recommended limits (0.5 mg/kg) being recorded. The data indicated moderate to high contamination for MOSH, from 16.5 mg/kg to 77.3 mg/kg, while average values below the limit of quantification (< LOQ) were highlighted for mineral oil aromatic hydrocarbons (MOAH) content. Based on the results, was difficult to establish a clear relationship between feed contamination, crop location and different pollution sources. However, the information obtained by assessing the relationship between feed contamination and pollution, indicated that the pollution sources from the plotting area had an important contribution to the contamination of the analyzed feedstuffs.

Key words: *evolution, milk production, NW Region, Romania, trends.*

INTRODUCTION

In recent years, the characterization of the hazards associated with environmental pollution has focused on different modern pollutants, thus studies on the assessment of the exposure of environmental elements to modern pollution have seen important progress.

Mineral oil hydrocarbons (MOH) are a complex mixture of contaminants derived from chemical products from crude mineral oils and other solvents, following extensive distillation and refining processes (IARC, 2012), which resulted in various residual fractions, including fuels (diesel, jet fuel), motor oils, lubricants; MOH can be also synthetically produced from coal, natural gas or biomass (Zoccali et al., 2016; Bratinova & Hoekstra, 2019).

As a result of their chemical origin, the presence of MOH in environmental substrates represents a serious threat to the environment, as mineral oils come from a comprehensive chemical group, with complex structures and a varied number of carbon atoms (n-C_{10-50}).

According to EFSA (2012); Van Heyst et al. (2018), the complexity of each type of mineral oil include two main fractions that can be analytically separated: MOSH-saturated hydrocarbons from mineral oils, composed of alkanes (normal, isoalkanes and cycloalkanes), which includes, generally, a complex of paraffins and naphthenes, and MOAH - aromatic hydrocarbons from mineral oils, comprising alkylated fractions and non-alkylated cyclic systems, respectively polyaromatic compounds substituted with alkyl groups in the n-C_{10-50} range.

The non-polar character given by their chemical structure makes MOSH & MOAH to have a special affinity for fatty tissues; for this reason, their presence has been confirmed in numerous types of vegetable oils (Nestola, 2022; Menegoz Ursol et al., 2023) and other products with high fat content (Zhang et al., 2019; Bauwens et al., 2022; Srbinovska et al., 2023). The occurrence of MOH and possible sources of contamination have been extensively described in the literature (Purcaro et al., 2016; Gharbi et al., 2017).

Regarding the incidence and frequency of occurrence, MOH can appear as a result of contamination and various intentionally uses in the production chain (Grob, 2014; Bruhl, 2016; Zoccalli et al., 2016; Canavar et al., 2018) through the products used for machinery and equipment (oils, lubricants) or as a component of packaging and transport materials (jute bags, cardboard packaging, waxed paper). More than contaminants, MOSH & MOAH are among the most important modern pollutants originating especially from emissions from urban area (transport, chemical and petrochemical industry), and also intensive agricultural processes (Van Heyst et al., 2018).

MOH contamination was mentioned for the first time around the 90s, when the first studies regarding the ecotoxicological safety and the origin of MOH contamination of different substrates has appeared (Grob et al., 1991; Moret et al., 1996; Wagner et al., 2001; Neukom et al., 2002). Since 2009, the analysis of MOH has become a routine through applied toxicological analysis methods. Biedermann et al. (2009) had published the first updated LC-GC-FID method that focused on the confirmation of MOSH and MOAH in different samples (Moret et al., 2016; Hochegger et al., 2021).

Although the effects on human or animal health are uncertain and insufficiently studied, the determination of MOH has gained more interest in recent years. For both, humans and animals, food is the main source of contamination of the body with MOSH and MOAH (Sdrigotti et al., 2021). Research carried out so far on animal tissues (Griffis et al., 2010; Boogaard et al., 2012; Barp et al., 2017; Cravedi et al., 2017) and human tissues (Barp et al., 2014; Biedermann et al., 2015; Adenuga et al., 2017; Carrillo et al., 2019) reported the ability of MOSH to accumulate especially in organs (liver, spleen) or in adipose tissues.

If for MOSH, according to the experiences carried out on animals and based on their structure and molecular weight, it has been shown that contamination can generally induce liver diseases, about MOAH, there are greater concerns given the aromatic structure, similar to polycyclic aromatic hydrocarbons (PAHs), with an alkylation degree of over 98% (Bratinova & Hoekstra, 2019) which thus imposes on them a genotoxic character and mutagenic actions, acting as promoters tumors (EFSA, 2012; Grob, 2018; Van Heyst et al., 2018; EFSA, 2019).

Despite the importance of feed for animal growth and development and for obtaining productions, and also the fact that the presence of MOSH & MOAH would represent a serious threat to feed and food safety considering the negative effects they could give, in the literature, there is no solid data on MOH contamination of feed are available to date.

Based on this background, the aim of this research was to quantify and confirm, for the first time, the presence of MOH in the feed representing the ration of a dairy farm, as well as to investigate the contribution of modern pollution sources to the final contamination level. Thus, processes of microwave assisted saponification (MAS) (Srbinovksa et al., 2021), followed by extraction step, modified according to Nestola & Schmidt (2017), were applied for 8 types of feeds, the samples thus prepared being analyzed by means of LC-GC-FID, having as a reference the method adapted according to Bauwens et al. (2022); Srbinovka et al. (2022).

The results from this work will contribute to the improvement of knowledge about MOSH and MOAH contamination of feed in relation to modern pollution, being a new topic for Romania and for the field of animal nutrition. Moreover, the results will create an overview of the understanding the importance of MOSH and MOAH contamination in animal nutrition and the importance of feed safety as an essential factor for the safety of animal production.

MATERIALS AND METHODS

Sample and sample collection

In order to quantify the level of contamination with MOSH and MOAH, were analyzed eight types of feed samples (raw materials and combined feed), taken from the feed base of a dairy cow farm located in an urban area in NE Romania, considered the second most polluted urban settlement in the country.

Excepting two samples: soybean meal (SM) and brewer's spent grain (BSG), which was purchased from external sources, all samples were obtained in the own vegetal farm of the unit, so that the feed sampling was carried out both directly, from the field, during harvesting, as well as from the storage area, with the necessary precautions to prevent any kind of modification or contamination.

The samples were collected in relation to the size of the sampling lots, in average quantities between 1-2 kg (depending on the type of sample). The selected samples were obtained by reducing the collective samples, according SR EN ISO 6497:2005 standard and Regulation (EC) 152/2009-Annex I.

Depending on the type of the feed, all the samples collected were prepared for analysis according to the guidelines mentioned in SR EN ISO 6498:2012 standard and Regulation (EC) 152/2009-Annex II, so as to avoid the contamination of the samples or the changes on their composition, using equipments that do not cause heating of the product mass or that do not be a source of contamination with mineral oils.

The feed samples were prepared for analysis according their type: cutting to 1-2 cm sizes, drying (moisture 8-12%) and grinding with the Grindomix GM 200 mill to fine powder. The samples were stored in aluminium packages until the analysis was carried out.

All the samples had a fat content < 4% and did not have in their composition other ingredients that could influence the results of the analyses. More information about the collected samples (types, quantities, plots) was included in Table 1.

Table 1. Characteristics of the samples and of the samples sites

Sample code	Area (ha)/ No.	% ration	Technological works: Phytosanitary (P) & Fertilising (F) treatments		Technological works: Harvesting	Technological works: Storage	Sampling sites location: d - roads/ trafic*	Sampling sites location: d - urban areas (no.)
Own production								
AH Alfalfa hay	30/6	5.5	Organic / Chemical Mechanica: sprinkler pump	F: Complex 16-16-16 (250 kg/ha) P: Corum (1.2 L/ha)	Mechanized/ (a) mowing and harvesting; (b) transport, (c) baling and wrapping press	Bales with plastic foil	~ 1 km / intense ~ 1 km/ medium**	~ 2 km (507.100)
AS Alfalfa silage		10.9			Mechanized/ (a) combine harvester; (b) transport; (c) crawler tractor	Concrete cell, polyethylene foil		
CS Corn silage	50/10	45.5		F: urea (100 kg/ha); NPK 20-20-0 (100 kg/ha); Ammonium nitrate (150 kg/ha) P: Henik (1.5 L/ha); Mustang (0.6 L/ha); Adengo (0.4 L/ha)			~ 4 km / intense ~ 1 km/ medium**	~ 2 km (507.100)
GrC Grain corn	23/4	6.4			Mechanized/ (a) combine harvester; (b) transport	Grain silos	~ 1 km / intense ~ 1 km/ medium**	~ 2 km (507.100)
T Triticale	8/2	4.5		F: urea (150 kg/ha); Ammonium nitrate (150 kg/ha); Lebosol (1.5 L/ha); P (I): Pixxaro Super (0.3 L/ha) P (II): Orius, Falcon Pro (0.5 L/ha) P (III): Mospilan (0.15 L/ha)		Grain silos	~ 5 km / medium	~ 4 km (2.067)
MF *Mixed feed*	-			Σ FL, SSL, SP, PB, SS, T, BB	Mechanized: technological trailer	-	∑ AH, AS, CS, GrC, T, SM, BSG	
External purchase								
SM *Soya meal*	-	18.2	-		Mechanized: transport	Grain silos	~ 1 km/ intense	~ 2 km (507.100)
BSG *Brewer Spent Grain*	-	6.5	-			Concrete platform	~ 1 km/ intense ~ 1 km/ medium**	~ 2 km (507.100)

Instrumentation and analysis conditions

For analysis of each carbon fraction: MOSH (n-C_{10-16}; n-C_{16-20}; n-C_{20-25}; n-C_{25-35}; n-C_{35-40}; n-C_{40-50}) and MOAH (n-C_{10-16}; n-C_{16-25}; n-C_{25-35}; n-C_{35-50}), for all the samples, a method based on LC-GC-FID was applied, preceded by a microwave-assisted saponification (MAS) step. The optimization of the method and the development of protocol belong entirely to the Food Chemistry Laboratory of the University of Udine (Department of Agrifood, Environmental and Animal Sciences), adapted and modified according to Biederman et al. (2009), Bierdemann & Grob (2012), and meets the requirements of JRC Guide (Bratinova & Hoekstra, 2019). The starting point in optimizing the method was the protocol developed by Moret et al. (2016), the method being subsequently applied with good results in numerous works (Menegoz Ursol et al., 2022; Srbinovska et al., 2022; Srbinovska et al., 2023). With some modifications, considering the specificity of the samples, the protocol corresponds to the method previously applied by Bauwens et al. (2022) for the determination of MOSH and MOAH in fish feed.
As a result of the interference of olefins, additional steps of clean-up of the samples (epoxidation, alluminium oxide clean-up) were necessary, being carried out according to the protocol described by Nestola & Schmidt (2017); CEN (2016).

RESULTS AND DISCUSSIONS

Regarding the level of contamination with MOSH and MOAH, in order to quantify and confirm the presence of MOH in relation to the modern sources of pollution, associated with the geographical area studied, were analyzed eight types of feed taken from plots located in one of the most polluted areas of the country (according World Air Quality Index, 2023).
Due to the complexity of the analyzed matrices, the monitoring and quantification of MOH proved to be difficult and required complementary analysis steps. However, the data obtained clearly indicated considerable MOH contamination for an important proportion of the analyzed feed samples.
By integrating the peaks in accordance with the principles and performance criteria established by the JRC Guide (Bratinova & Hoekstra, 2019) in correspondence with the European Commission (EC, 2022), was achieved the quantification for each carbon fraction included in the n-C_{10-50} range (6 MOSH C-fractions; 4 MOAH C-fractions) using CyCy as internal standard for MOSH quantification and average of 5B, 1-MN, 2-MN and TBB internal standards for MOAH quantification. Tables 2 and 3 summarizes the average values, expressed in mg/kg, showing the level of feed contamination with MOSH and MOAH for each carbon fraction (n-C) and for total content (n-C_{10-50}).

Table 2. MOSH content (mg/kg) of feed samples

Sample	% fat	IS MOSH	MOSH (mg/kg)						
			C_{10-16}	C_{16-20}	C_{20-25}	C_{25-35}	C_{35-40}	C_{40-50}	C_{10-50}
AH	1.67	CyCy	12.2	9.2	5.3	3.4	< LOQ (0.4)	< LOQ (0.4)	**31.0**
AS	0.55		9.9	10.1	8.1	11.0	1.5	1.0	**41.6**
CS	1.27		8.7	7.4	5.5	6.8	1.2	0.8	**30.5**
GrC	3.74		9.0	27.9	23.8	12.4	2.1	2.1	**77.3**
SM	1.08		4.1	4.1	3.0	5.0	< LOQ (0.2)	< LOQ (0.1)	**16.5**
T	1.37		2.3	3.2	2.0	12.5	4.7	3.3	**28.0**
BSG	2.24		14.6	8.4	8.5	27.2	1.5	0.7	**60.9**
MF	1.08		7.3	10.8	11.9	23.2	3.3	1.9	**58.4**

Absence of data labels (nd.) indicates levels below the LOQ (0.5 mg/kg for MOSH); CyCy = cyclohexylcyclohexane.

Table 3. MOAH content (mg/kg) of feed samples

Sample	% fat	IS MOAH	MOAH (mg/kg)				
			C_{10-16}	C_{16-25}	C_{25-35}	C_{35-50}	C_{10-50}
AH	1.67	5B/ 1-MN/ 2-MN/ TBB	< LOQ (0.06)	0.98	< LOQ (0.09)	< LOQ (nd.)	**1.13**
AS	0.55		< LOQ (0.09)	1.71	< LOQ (0.20)	< LOQ (nd.)	**2.00**
CS	1.27		< LOQ (nd.)	0.47	< LOQ (nd.)	< LOQ (nd.)	**0.47**
GrC	3.74		0.75	3.77	< LOQ (0.16)	< LOQ (nd.)	**4.68**
SM	1.08		0.24	0.73	< LOQ (nd.)	< LOQ (nd.)	**0.97**
T	1.37		< LOQ (nd.)	< LOQ (nd.)	< LOQ (nd.)	< LOQ (nd.)	**< LOQ**
BSG	2.24		< LOQ (0.20)	2.77	< LOQ (0.15)	< LOQ (nd.)	**3.13**
MF	1.08		< LOQ (nd.)	< LOQ (0.49)	< LOQ (0.02)	< LOQ (nd.)	**0.51**

Absence of data labels (nd.) indicates levels below the LOQ (0.5 mg/kg for MOAH); 5B = n-pentylbenzene; 1-MN = 1-methylnaphthalene; 2-MN = 2-metilnaftalenă; TBB = 1,3,5-tri-terț-butil-benzen.

Different from a compositional point of view, all analyzed feed samples, alfalfa hay (AH), alfalfa silage (AS), corn silage (CS), grain corn (GrC), soybean meal (SM), triticale (T), brewers spent grain (BSG) and mixed feed (MF) recorded MOSH concentrations above the limits recommended by the Guide JRC and the European Commission (2022) for products with a fat content < 4%, respectively 0.5 mg/kg. The data obtained indicating a moderate contamination, from 16.5 mg/kg (min. value) to a particularly high contamination 77.3 mg/kg (max. value). Instead, values below the limit of quantification (< LOQ) were highlighted regarding the MOAH content for the analyzed samples, 50 % of the samples recording values above the safety limits recommended previously; the highest MOAH contents were confirmed for BSG samples, 3.13 mg/kg and GrC samples, 4.68 mg/kg. The chromatograms in Figure 1 confirm the presence of MOSH and MOAH in the feed samples analyzed. Also, Figure 2 shows the contamination profile of the feed samples, by carbon fractions (n-C), that indicate the same hump on the same molecular rank, n-C_{10-35} for MOSH and n-C_{16-25} for MOAH, considered typical of contamination with lubricants, engine oil or hydraulic oil, as Srbinovska et al. (2023) mention in the similar study on MOH contamination of basil pesto.

However, the typical contamination was not considered representative for the high level of contamination found, so, that sources of contamination of a different origin than those already anticipated were associated with the analyzed fodder.

The literature generally shows that the presence of hydrocarbons from mineral oils as contaminants can come from various sources, from urban areas, car traffic or industrial sites (Neukom et al., 2002), but also from technological operations in farms (EFSA, 2012).

The information previously collected in the sampling sheets developed by Matei & Pop (2022) show that the farm is located in an urban area, in the immediate vicinity of numerous sources of pollution from industrial activities, construction and road infrastructure, municipal waste or road and air traffic, so that the pollution risk highlighted can be a probable cause of the high level of contamination with MOSH and MOAH identified for the analyzed feeds.

From the samples obtained in the own vegetable farm (AH, AS, CS, GrC, T, MF), excepting triticale samples (that will be discussed separately), all the other samples correspond to similar cultivation areas, being exposed to the same sources of pollution. Samples of alfalfa, corn for silage and for grains harvested near roads with high traffic (approx. 20.000 cars/day; Traffic Management Office, Iasi), air traffic runways (25 landing-takeoff cycles/day) and near areas of industrial activities (~1-2 km) contained average levels of MOSH above 30 mg/kg and MOAH above 1 mg/kg.

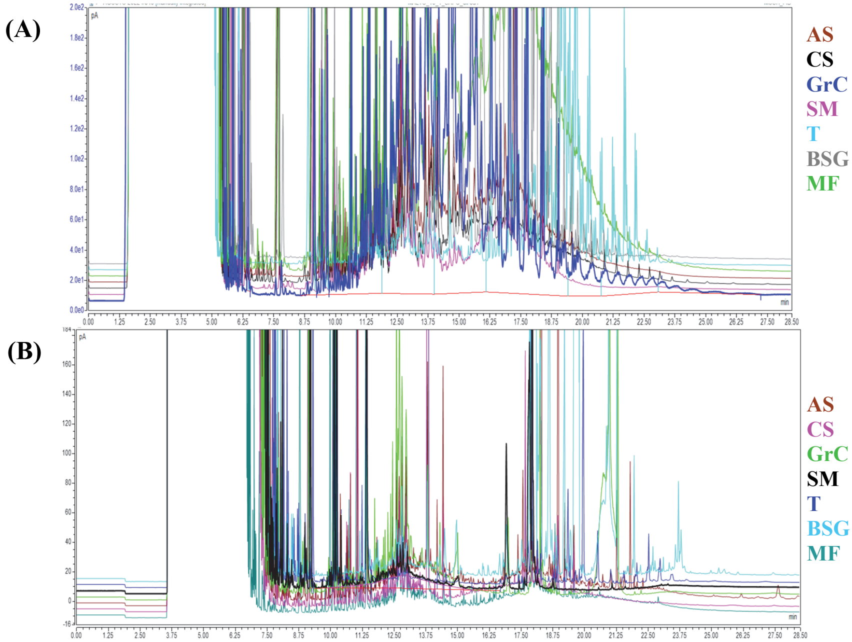

Figure 1. Overlay of MOSH (A) and MOAH (B) chromatograms of feed samples.
The figure legend is reffered the interpretation of the references to colour

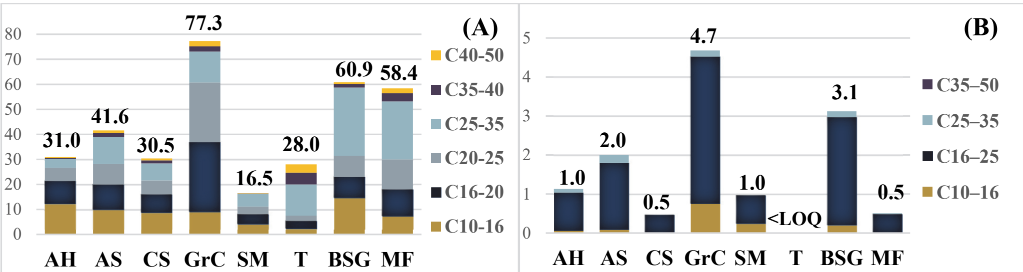

Figure 2. MOSH (A) and MOAH (B) distribution on C-fractions in feed samples

However, in terms of MOSH and MOAH concentrations detected for feed samples exposed to the same pollution sources, no uniformity was observed; giving an example, the MOSH and MOAH content for GrC samples (77.3 mg/kg and 4.68 mg/kg), was double that the content shown for AS (41.6 mg/kg MOSH, 2.0 mg/kg MOAH), AH (31.0 mg/kg MOSH, 1.13 mg/kg MOAH) or CS (30.5 mg/kg MOSH, < 0.5 mg/kg MOAH). These can be argumented bu the higher fat content of feed samples, therefore a higher concentration of contaminants in these types of samples, which can confirm the hypothesis developed by Matei et al. (2022) regarding the contribution of fats in the contaminant accumulation process. Also, the potential contamination of corn samples during processing cannot be keep out.

Figure 3 shows a selection of feed samples whose chromatograms obtained from contaminant analysis with LC-GC-FID system confirm the presence of MOSH and MOAH. The most representative samples were selected, with the most important levels of contamination (GrC; MF) and whose MOSH traces highlight the typical contamination profile previously mentioned. Carbon fractions with important contamination areas was also highlighted, but still unknown in origin, thus suggesting multiple contamination.

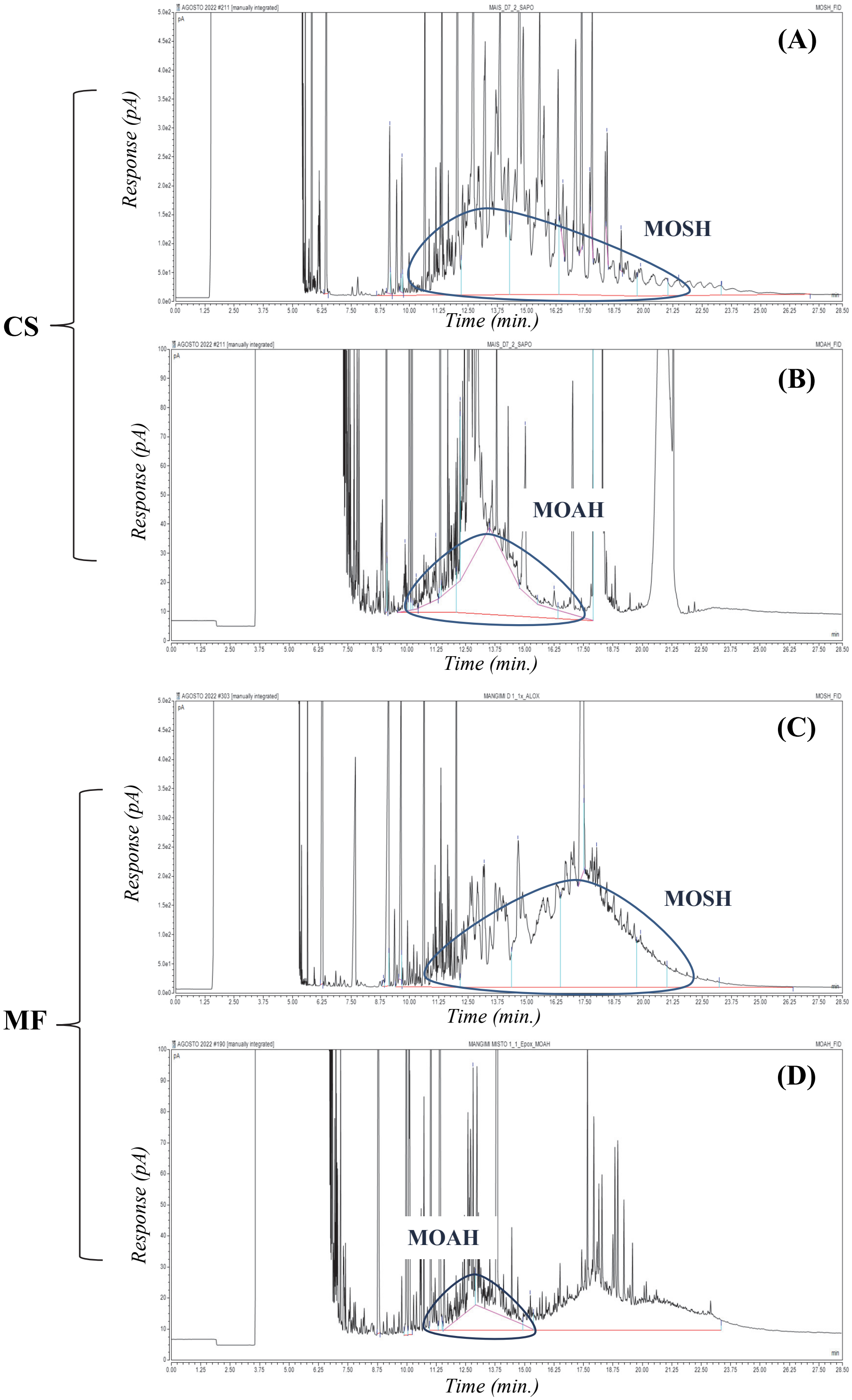

Figure 3. LC-GC-FID chromatograms of a selection of samples with existing contamination (CS, MF). (A), (C): MOSH traces and (B), (D): MOAH traces

Contrary to the previously samples, the T (triticale) samples located further from potential sources of pollution (the plot located in a rural area with low traffic density, ~ 5 km from populated areas), revealed an unexpectedly high level of MOSH contamination (28.0 mg/kg) whose origins still remain unknown. For MOAH instead contents below < LOQ were observed, which allows establishing a clear relation regarding the location of the crop relative to the sources of environmental pollution.

Regarding the compound feed samples, 58.4 mg/kg MOSH and 0.5 mg/kg MOAH concentrations obtained was considered proportional to the contribution of the raw feeds to the mixture and the content of MOSH and MOAH obtained for them.

Although they are outsourced samples, for which there is no information on crop location and associated potential pollution sources, the level of MOSH and MOAH contamination of SM (16.5 mg/kg MOSH; 1.0 mg/kg MOAH) and of BSG (60.9 mg/kg MOSH; 3.1 mg/kg MOAH) should not be considered minor as they indirectly contribute to the contamination level of the mixture formed.

The information obtained by evaluating the relationship between feed contamination and pollutant factors clearly indicated that the pollution sources associated with the analyzed sampling areas had an important contribution to the contamination of the analyzed feed. In addition, the possible contamination during feed care, harvesting and storage operations or the contribution of undeclared work practices by farmers that can substantially contribute to the contamination of feed with MOSH and MOAH have not been completely neglected.

CONCLUSIONS

In both MOSH and MOAH analysis, good quantitative and confirmatory results regarding contamination were generally obtained, important to prove the reliability of the LC-GC-FID detection method used, for which good performance was demonstrated (LOQ), according the requirements of JRC guideline.

Chromatographically, the contamination profiles confirmed the presence of different sources of pollution and contamination. In relation to the exposure to different sources of pollution founded, important clues were assigned to pollution from the urban area. The level of contamination observed was considered proportional to the degree and intensity of exposure to the associated modern pollution sources (urban traffic, air traffic, industrial activities). However, establishing a clear relation between the level of feed contamination, crop location and proximity to different sources of pollution has proven difficult.

In terms of identifying the most important sources of pollution and contamination, great efforts are needed to minimize contamination, although awareness of existing problems, and using good manufacturing practices can help to reduce the risks associated with high levels of contamination.

More studies are needed to assess the contribution of modern pollution to the contamination of feed with MOSH/MOAH. Although the contamination of feed with MOSH and MOAH does not currently cause significant problems, the potential cumulative risk may be sufficiently high, therefore, the economic consequences and the potential negative consequences on animal health and on the safety of consumers should not be considered minor.

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RESEARCH ON THE EFFECT OF SOME NUTRITIONAL SUPPLEMENTS ON QUANTITATIVE AND QUALITATIVE PARAMETERS OF GOAT'S MILK

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Abstract

The purpose of this study was to evaluate the influence of the administration of nutritional supplements on the quantity and quality of goat's milk. For this purpose, homogeneous groups of goats from the Carpathian breed, in the middle of the lactation period (n = 10), were created, to whom were administered in food, experimentally: hemp seeds (group 2), hemp seeds and mineral supplement (group 3), flax seeds (group 4), flax seeds and mineral supplement (group 5). Milk production and milk protein and fat percentages were monitored for 20 days, and the results obtained were compared with those of the control group (group 1). The obtained results showed that in terms of milk production, there were significant increases (p<0.05) between the control group and the experimental groups (with 6.95% for group 3 and by 5.42% for group 5). Regarding the percentage of proteins, significant increases were observed in the case of groups 3 (6.06%) and group 5 (9.09%). Regarding the percentage of fat, it increased in the case of all experimental groups, the increases being 4.65% (p<0.05) for group 2, 11.62% (p<0.01) for group 3, 6.97% (p<0.05) for group 4, and 9.30% (p<0.01) for group 5.

Key words*: flax, goat, hemp, milk, supplement.*

INTRODUCTION

Knowledge about the physiology of lactation has made significant progress recently, constituting a priority direction of research in the zoo veterinary field. This matter has been accentuated by the presence of milk in human nutrition and the food industry as a raw material (Savu et al., 2002; Răducuţă et al., 2008; Visoescu et al., 2015; Petcu et al., 2022). Therefore, the knowledge referring to humoral regulation of the lactation process has expanded, which led to the description of multiple hormones that intervene in physiological conditions, at well-determined moments, and have a capital role in mammogenesis, lactogenesis, and galactopoiesis (Codreanu, 2018), phyological processes closely related to the reproduction activity (Bociu et al., 2015), and to the milk composition variations, influenced by internal or external factors (Petcu et al., 2022). Furthermore, it has been proved the possible usage of some hormonal entities, like GH, LTH, estrogen, progesterone and placental lactogen, in the manipulation and optimisation of mammogenesis, lactogenesis, and galactopoiesis (Cotor at al., 2011). However, the possible adverse effects of artificially administered hormones or growth promoters and the negative implications they could have on the quality and sanitation of milk and derived products (Goncearov et al., 2004; Ionita et al., 2016; Petcu et al., 2020; Pogurschi et al., 2022a; Pogurschi et al., 2022b; Ghimpeteanu et al., 2022) and, implicitly, in human health, raised certain question marks on the feasibility of implementing such biotechnologies in animal husbandry. Currently, it is known that goat milk production is strongly influenced by the growth system (Răducuță et al., 2015), as well as the size of the flocks and the quality of individuals from a farm (Răducuță et al., 2010).

At the same time, research in the field of lactation physiology has highlighted the involvement of essential food components in the biosynthesis of milk components and their importance in ensuring efficient galactopoiesis. In this sense, the importance of the protein content of the food ration and their quality (the presence of essential aminoacids in the food) in ensuring a decresed production of milk, with an improved protein content, has been demonstrated (Chilliard et al., 2002). It was also demonstrated the importance of cellulose in ensuring an optimal environment for the development of the cellulolytic flora, at the level of the ruminal microecosystem. This is involved in the synthesis of volatile fatty acids (VFAs), the raw material used in the pre-metabolic phases of the biosynthesis of milk lipids (biosynthesis of saturated, short-chain fatty acids) at the level of mammary acini (Cotor, 2015).

The administration of nutritional supplements based on oleaginous plants, which contain high levels of long-chain unsaturated fatty acids, especially linoleic acid in particular, have shown positive effects on the level of milk fat and its quality (Chouinard et al., 2001; Cotor et al., 2009). In this sense, research was undertaken on the effect of supplementing the food ration with: seeds, cakes and soybean oils, olive oil, sunflower oil, etc., on the level of ruminal synthesis of VFA and the balance of fatty acids in milk (Givens et al., 2003; Harvatine et al., 2006).

At the same time, a series of additives based on substances of mineral and animal origin are known that have an effect of inhibiting bacterial cellulase and implicitly reducing the concentration of resulting acetic acid, a fact that can lead to a decrease in the weight of short-chain fatty acids in the composition milk fat (Harvatine et al., 2005; Kholif et al., 2015; Morsy et al., 2015).

Research in this sense has been and continues to be carried out, trying to identify optimal ways of administering such additives, under conditions of economic profitability. An untested oleaginous ingredient for the purpose mentioned above is represented by hemp seeds (*Cannabis sativa*). Along with hemp, flax seeds (*Linum usitatissimum*) are noteworthy for their high content of unsaturated fatty acids, mentioned above.

The administration of inorganic alkalizing substances (sodium bicarbonate, MgO), in parallel with fats of vegetable origin, reduced the negative effects on milk production and the percentage of proteins implied by supplementing the feed ration with fats, in ruminants (Martini et al., 2004). Thus, a mineral supplement, with a recipe (75% sodium bicarbonate, 10% magnesium oxide and 15% calcium carbonate) that will quantitatively and qualitatively improve the lipid content of milk, without affecting at the same time, in a way significant, protein content and milk production as a whole, was constituted in an objective approach of the present work.

Nowadays, the administration of mineral supplements is a common practice in many areas of animal husbandry (Ghiță et al., 2021).

MATERIALS AND METHODS

A number of 50 lactating goats, from the *Carpathian breed*, aged between 3 and 5 years, in the same phase of the lactation period, were used for the experiment. The animals came from the same holding, benefiting from identical maintenance conditions. It was also considered that the animals in the experimental group have the same state of maintenance, because the excess development of adipose tissue can affect the functions of lymphocytes (Ghiță et al., 2021), making the animals susceptible to diseases. The experiment took place over a period of 20 days, on healthy animals that were not administered any medication, as the effect of anti-inflammatory medication on milk production (Codreanu, 2018) and the immune system (Ghita et al., 2015) is known.

Five experimental groups (of 10 animals each) were organized, which were treated in the manner described in Table 1. Food additives (hemp seeds, flax seeds and mineral supplement) were administered daily and individually, being incorporated into the food portion. Daily milk production per goat was measured throughout the experiment. On days 1, 5, 10, 11, 15 and 20, milk samples were collected in order to determine the protein and fat concentration.

Table 1. The method of administration of the tested additives, in the experimental groups

Group number	**Hemp seeds g/goat/day**	**Flex seeds g/goat/day**	**Mineral supplement (75% $NaHCO_3$, 10% MgO and 15% $CaCO_3$) g/goat/day**
Group 1 (n=10)	-	-	-
Group 2 (n=10)	200	-	-
Group 3 (n=10)	200	-	100
Group 4 (n=10)	-	200	-
Group 5 (n=10)	-	200	100

The protein and fat content were determined using a Lactoscan 60 LCD Milk Analyzer (IR spectrophotometry).
The statistical analysis consisted in calculating the mean and the standard error.
At the same time, in order to determine the significance of the differences between the experimental groups, the t-test (Student test) was applied.

RESULTS AND DISCUSSIONS

The results obtained in the present study indicate that the groups that received only hemp and flax seeds in the ration (groups 2 and 4) did not have higher milk production than the control group, in the two experimental stages.
The average daily production of milk per lot is presented in Table 2.

Table 2. Average daily milk production per group and the experimental phase

Group	Average daily milk production/group, in the first 10 days of the experiment (ml)	Average daily milk production/group, in days 11-20 of the experiment (ml)
Group 1 (n=10)	1420	1438
Group 2 (n=10)	1452	1468
Group 3 (n=10)	1461	1538*
Group 4 (n=10)	1452	1454
Group 5 (n=10)	1494	1516*

*P<0.05

In the first period of the study (days 1-10), the average values obtained did not show significant increases in milk production, while, in the second period of the study (days 11-20), groups 3 and 5, whose ration of was supplemented with flax or hemp seeds and mineral supplement, showed an intensification of milk production, compared to the control group, namely, group 3 with 6.95% and group 5 with 5.42%, the differences being significant in terms of statistical view ($P<0.05$).
Studying the specialized literature (Martínez et al., 2011; Kholif et al., 2015) it is found that the results obtained in the present study were similar to the results of other scientific researches. Nutritional supplements were administered in the present study in order to study their effect on milk quality. The slight increase in milk production in the case of groups 3 and 5 can be explained by the influence of the mineral supplement that buffered the ruminal pH facilitating the activity of the symbiont flora at that level. In this way, the digestion processes were influenced in a positive way, which can also lead to an increase in milk production.
The average protein concentration of the milk samples, collected from the experimental groups, is presented in Figure 1.
The obtained results indicate that the groups that received only hemp and flax seeds in their rations (groups 2 and 4) did not have much higher protein concentrations than the control group, in any of the two experimental stages.
Groups 3 and 5, whose ration was supplemented with flax or hemp seeds and mineral supplement, showed an increase in the milk protein concentration (group 3 with 6.06% and group 5 with 9.09%) compared to the control group, the differences being statistically significant ($P<0.05$).

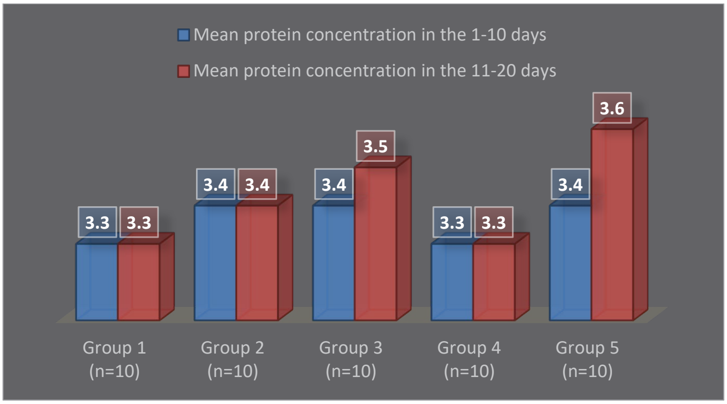

Figure 1. Average protein concentration in milk samples, per group and the experimental phase

In the specialized literature (Martínez et al., 2011; Kholif et al., 2015) results similar to those of the present study are reported. The explanation is represented by the fact that the nutritional supplement administered in the present study had a buffering effect on the ruminal pH, offering better conditions to the proteosynthetic microbial flora at the ruminal level. In this way, the protein level of the ruminal content increased, implicitly leading to an increase in aminoacidemia and finally in milk proteins.

The average fat concentration of the milk samples collected from the experimental groups is presented in Figure 2.

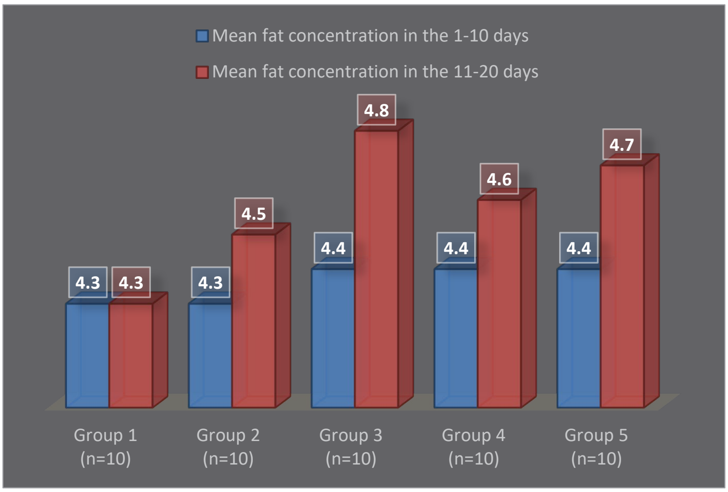

Figure 2. Average fat concentration in milk samples, per group and the experimental phase

The obtained results indicate that the groups that received only hemp and flax seeds in the ration (groups 2 and 4) recorded higher lipid concentrations than the control group, by 4.65%, in the case of group 2 and by 6.97%, in the case of group 4, the differences being statistically significant ($P<0.05$).

Groups 3 and 5, whose ration was supplemented with flax or hemp seeds and mineral supplement, showed an increase in the lipid concentration of milk (group 3 with 11.62% and group 5 with 9.30%) compared to the control group, the differences being distinctly significant from a statistical point of view ($P<0.01$).

In the specialized literature (Reklewska et al., 2002; Chilliard et al., 2002; Martínez et al., 2011; Morsy et al., 2015) similar results to those of the present study were reported. The increase in milk fat percentage is due to the increased food intake, the experimentally administered oilseeds being rich in unsaturated fatty acids. Also, the experimentally administered mineral nutrient supplement prevented the acidification tendency of the rumen pH (due to the production of VFAs), providing optimal conditions for the action of the symbiotic microflora in the rumen.

CONCLUSIONS

The groups that received only hemp and flax seeds in the ration, did not record milk production superior to the control group.

The groups whose ration was supplemented with flax or hemp seeds and mineral nutritional supplement showed an intensification of milk production (group 3 with 6.95% and group 5 with 5.42%) compared to the control group, the differences being statistically significant.

The groups that received only hemp and flax seeds in the ration did not record higher protein concentrations than the control group.

The groups whose ration was supplemented with flax or hemp seeds and mineral nutritional supplement, showed an increase in the milk protein concentration (group 3 with 6.06% and group 5 with 9.09%) compared to the control group, the differences being significant from statistical point of view.

The groups that received only hemp and flax seeds in their ration recorded higher lipid concentrations than the control group, by 4.65%, in the case of group 2 and by 6.97%, in the case of group 4, the differences being statistically significant. Groups 3 and 5, whose ration was supplemented with flax or hemp seeds and mineral nutritional supplement, showed an increase in the lipid concentration of milk (group 3 with 11.62% and group 5 with 9.30%) compared to the control group, the differences being statistically significant.

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THE FUNCTIONAL APPROACH FOR THE STUDY OF CROP PEST PREDATORY ARTHROPODS

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Abstract

Crop pests represent the sole cause of pesticide use in agroecosystems, a practice linked to pollution and health problems. The use of natural enemies against crop pests can prove to be a solution to partially alleviate this anthropic pressure and reduce associated costs. Predatory arthropods, such as spiders and carnivorous ground beetles can be efficiently used to reduce harvest consumption by pests. The community structure and overall fitness of these natural enemies (and their efficacy in pest control) are inextricably linked to the functional traits of each comprising taxa. The trait-based approach proposes functional traits and functional diversity indices, as the common denominators between biotic communities instead of taxonomic diversity. This approach for the study of predatory arthropods in agricultural ecosystems, especially in the interest of mediating production losses, has recently increased in popularity due to its ease of use for biotic communities' assessments and for its convenience in predicating ecosystem interactions and fluxes. The scope of this research is to critically analyse the current state of knowledge on the use of the trait-based approach to study predatory arthropods in agroecosystems, in order to provide guidelines of how this framework can be efficiently used for scientific research and ecosystem management and to identify existing knowledge gaps, in order to support future scientific endeavours.

***Key words**: agricultural ecosystems, functional diversity, functional traits, natural enemies, predatory arthropods.*

INTRODUCTION

The imminent increase of worldwide human populations represents one of the main challenges for the continued existence of the current socio-economical system and for the actual functional stability of the ecosphere. The role of agriculture in maintaining social stability is expected to increase with the growing demand for food and so is the impact that it will have on the biodiversity of the world's terrestrial ecosystems. Aside from their main use for crop production, agricultural landscapes also host part of this biodiversity by providing habitat for multiple biotic communities, some of which provide beneficial ecosystem services, such as pollination (Lonsdorf, 2009) and pest control (Alexandridis et al., 2021) and which are usually affected by intensive agronomic practices (Bianchi et al., 2006; Deppe & Fischer, 2023).

Predatory arthropods inhabiting agricultural ecosystems, such as spiders and carnivorous ground beetles, represent a viable alternative to conventional pesticide use that can be used to control the populations of crop pests (Holland, 2002; Kromp, 1999; Marc et al., 1999; Sunderland, 1999). Agricultural practices and landscape management directly influences the habitat of natural enemies and their efficiency to provide the ecosystem service they are valued for (Kromp, 1999; Sunderland & Samu, 2000). Patch and landscape scale characteristics shape the communities of natural enemies in multiple ways, most of which are still not completely understood (Alexandridis, et al. 2021). The community structure, overall fitness and pest control efficiency of natural enemies are all inextricably linked to the functional characteristics of these organisms, termed functional traits or just traits (Deppe & Fischer, 2023; Maas et al., 2020). They represent generalizable properties of organisms that dictate how individuals interact with their environment and that apply for all species, thus bypassing taxonomic barriers (Moretti et al., 2017; Wong et al., 2019).

The trait-based approach utilizes traits as the common currency used to compare biotic communities, in their regard to environmental stimuli and to their effect over ecosystem processes and services. Since predatory arthropods inhabiting agricultural landscapes are associated with the pest control ecosystem service, it is clear that the study of this taxa is conducted primarily from a functional perspective. The use of functional traits and measures of functional diversity holds promise in helping researchers understand the effects of different agricultural practices on natural pest control services. This is achieved by observing the functional changes of natural enemy communities at various scales and from a unified perspective. The use of the trait-based approach promises to increase our knowledge and understanding of processes associated with crop pest predators and to help improve agricultural practices and ecosystem management, both locally and at the landscape level.

This review aims to: (1) identify the main traits and functional diversity components that shape the capacity of spiders and predatory ground beetles to control crop pests populations (response variables); (2) identify the main pressures that act at patch and landscape scales (predictor variables) and their effects on the functional traits and diversity components of the aforementioned communities; (3) highlight the impact of these effects on the pest control service and provide suggestions on how to ameliorate them and (4) suggest future research directions based on the identified knowledge gaps in this field of research.

MATERIALS AND METHODS

In order to find relevant articles related to the topic, the Web of Science Core Collection database was queried. The search sting used included a combination of terms related to the taxa (spiders and predatory ground beetles), habitat types (agricultural ecosystems and landscapes) and functional assessment metrics (functional traits and functional diversity components) of interest. The initial results of the query returned 865 articles, which were further screened in order to select only the studies that addressed the functional traits or functional diversity components of predatory arthropods in agricultural ecosystems.

The results sections were structured based on the functional traits and diversity components assessed, the level of the research (patch and landscape scale) and on the type of acting pressures (soil preparation; crop selection and rotation, pesticide use and landscape management). Firstly, the effect of the acting pressures on each trait was described, followed by discussions regarding possible improvement methods and, finally, possible future research directions were suggested, in order to further develop the field of research.

Table 1. Literature search sting

TOPIC: "predatory arthropod*" OR spider* OR beetle* OR arachnid* OR carabid*
AND
TOPIC: functional* OR trait*
AND
TOPIC: agricultur* OR agro*

RESULTS AND DISCUSSIONS

General findings

Based on the scientific literature screening, the most commonly used traits for the research of arthropod natural enemies were: feeding preference, body size and dispersal capacity. In most cases, these traits were studied together, with the first two being addressed based on their importance in pest control, while the latter due to its relevance for recolonization from adjacent habitats. Hunting strategy, fecundity and activity period were also studied, but to a lesser degree, prompting the current study to focus more on the three most addressed traits. Although the use of functional diversity is relatively novel, some valuable studies do exist. Based on the literature screening, the main pressures affecting the communities of natural enemies in agricultural landscapes are pesticide use (conventional vs. organic agriculture), soil preparation practices, such as tillage and winter cover crops and most importantly, the reduction of natural and seminatural habitat patches.

The overall findings suggest that the functional traits, functional diversity and capacity for pest control of natural enemies often varies between spiders and ground beetles and between habitat and landscape characteristics. More than often, research shows that multiple interacting factors at multiple scales act, in order to shape the functionality of the studied communities.

Furthermore, it is important to point out that research of many traits is still underdeveloped and that most of the research takes place in temperate climates (in Europe and North America), while the most drastic landscape changes caused by agriculture take place in the least studied tropical zones.

Pesticide use

Pesticide use associated with conventional agricultural practices is documented to reduce the body size of spiders and the abundance of web hunters within cropland communities (Michalko and Kosulic, 2020), while having the opposite effect in vineyards (Caprio et al., 2015). These findings underline the importance in understanding how different pesticides act in different agricultural habitat types. However, the body size of ground beetles was shown to decrease in organic orchards, as their dispersal capacity increased (Caprio et al., 2015; Mickael et al., 2015), a discovery which also shows the response variability among different taxa (reinforcing the need for multitaxon studies). In opposition to ground beetles, spiders decreased in body size in conventional orchards and increased in organic ones (Mazzia et al., 2015). Opposite to spiders from the same type of agricultural habitat, carabid body size was documented to increase in crops with reduced pesticide use (Galle et al., 2018; Rusch et al., 2013). The hunting strategy of natural enemies was shown to change with pesticide application from active hunters to web and ambush hunters (Caprio et al., 2015). Furthermore, conventional pesticide use was further documented to have daunting sublethal effects on spider assemblages, such as reducing predatory rate in both web hunters and ground hunters (Boyd et al., 2022; Deng et al., 2007; Deng et al., 2008) and causing a reduction in dispersal capacity and even paralysis of multiple spider taxa (Shaw et al., 2006). Interestingly enough, another recent study showed the increase in killing neonicotinoids contaminated prey by spiders, but without consumption (Korenco et al., 2019) This is a factor which can prompt short term increase in pest control, but a long-term reduction of this service, due to useless energy waste of the predators which could indirectly affect their overall fitness. Though no direct link between pesticide uses and body size of spiders was found, it can be argued that reduced mobility and hunting can slow down the development of spiders in agricultural landscapes, leading to a reduction in body size and highlighting their pest control efficiency.

The functional diversity and evenness of spider communities was recorded to increase in organic orchards (Mazzia et al., 2015). Pesticide use was documented to decrease the functional diversity of ground beetles (Galle et al., 2018) along with intensive tillage and bare soil practices (Muneret et al., 2002) and along with the reduction in habitat diversity at landscape scale (Gayer et al., 2019). Though much research is still needed, organic research seems to increase the functional diversity and pest control services standalone by also helping increase the diversity of adjacent natural and seminatural habitats (Caprio et al., 2015). These findings reaffirm the importance of habitat diversity in agricultural landscapes and the idea that the effect of different agricultural practices should be assessed in broader scale perspectives. Further search on pesticide use can focus on the nonlethal effect over the functional traits of natural enemies and of their prey. Furthermore, studies comparatively addressing the effect of various stimuli on agricultural habitats with conventional and organic agriculture could be very important to understand local and large-scale interactions between natural enemies and their environment in dynamic landscapes. More research on better biopesticides is needed. Overall, pesticides (even biopesticides) should be examined in regard to their morphological and behavioural effects on nontarget beneficial taxa and to their interacting trophic links, including natural enemies and pollinators (Cappa et al., 2022).

Tillage practices

A number of studies have shown that the body size of carabids seems to decrease with tillage intensity (Hanson et al., 2016; Jacobsen et al., 2022). However, the dispersal capacity of carabids was documented to increase in intensively managed crops, probably as an adaptation measure to the reduced body size and preying efficiency (Hanson et al., 2016). One research study points out that the effect of tillage on carabid body size and dispersal capacity seems to be season dependent and that lower intensity tillage (Non-Inversion Tillage) seems to favour capable dispersal taxa (Kosewska, 2016). Though less studied than ground beetles, spiders were also documented to respond to tillage, by an increase in the number of cursorial dispersers (Topa et al., 2021). The activity of spiders was documented to decrease with land management intensity in landscapes rich in natural and seminatural habitat patches and, conversely, to decrease in vineyards with natural management type (Pfingstmann, 2019). This aspect further solidifies the need for multiscale studies in the functional ecology of natural enemies.

The functional diversity and density of spiders were found to be lower in no-tillage fields, thus reducing aphid effectives (Tahir & Mukhtar, 2012). The effect of tillage on the functional diversity of ground beetles was found to be similar to spiders, with the highest functional diversity recorded in non-tillage sites (Jacobsen et al., 2022; Muneret et al., 2002). Tillage needn't be bad as some research found that reduced intensity tillage seems to reduce the effect of urbanization al landscape scales in comparison to conventional tillage, by harbouring more functionally diverse ground beetle communities in agricultural patches and surrounding habitats (Tamburini et al., 2016). Based on the last result, future research endeavours should focus on (1) the effect of low impact tillage in promoting biodiversity across landscapes and (2) the response of natural enemy communities from these habitats to other possibly acting pressures. Additionally, further research should focus on the impact of seasonal changes and predicted climatic changes on different crop management systems, in order to improve the conservation of beneficial invertebrates, such as natural enemies and pollinators and of the ecosystem services they provide.

Landscape management

Based on the literature screening, landscape management is by far the most addressed pressure analysed in the functional ecology research of natural enemies. Increased natural and seminatural habitats cover within agricultural catchments was documented to increase overall pest predation (Zamberletti et al., 2021). Habitat diversity is connected to different hunting strategies and better pest control efficiency. Web hunting spider were documented to be efficient against flying pest, ambushing pests against flower visiting pests, while active ground predators are inclined to prey on pest with developmental stages in and on the ground. (Benhadi-Marin et al., 2019). Multiple research document body size as an important trait related to pest control at landscape level, both in spider and ground beetles (DeLong & Uiterwaal, 2022). Increased body size of natural enemies was observed to be linked to habitat size (DeLong & Uiterwaal, 2022; Gale et al., 2020) and place within the filed (Gale et al., 2020). The availability of natural and seminatural habitat patches increased carabid and spider body size (Plath et al., 2021) and active hunting species (Maas et al., 2021). Furthermore, the body size of multiple natural enemies was recorded to increase in proximity to natural and seminatural habitats (Zhang et al., 2019; Zhang et al., 2020).

A number of research studies associated the community wide shift of dispersal capacity with both local and landscape drivers (Gale et al., 2020; Saquib et al., 2022). For spiders, the proximity to natural habitats such as forests (Saquib et al., 2022) and wildflower strips (Gale et al., 2020) was shown to increase ballooning propensity. Ballooning propensity also increased in the central parts of crop fields and the choice of structurally complex plant crops was shown to increase ballooning propensity. The community wide dispersal capacity of ground beetles increased in relation with patches of natural and seminatural habitats (Pecheur et al., 2020). The activity of both spiders and ground beetles was linked to increase with proximity to seminatural habitats (Jacobsen, 2022; Maas et al., 2021; Pecheur et al., 2020) and with their increased availability at the landscape level (Maas et al., 2021).

The availability of varied seminatural habitats (such as forest, meadows) at the level of agricultural landscapes is documented to further aid in natural pest control, by providing shelter for natural enemies. The necessity of integrating diverse natural and seminatural habitats at larger scales is important due to the functional differences exhibited by each type of natural enemy community (Michalko & Birkhofer, 2021). Recently, the importance of

these habitats was further demonstrated to vary seasonally depending on the type of migrating taxa and the type of habitat (Robinson et al., 2021), further reinforcing the need for large scale multi-seasonal studies in agricultural landscapes. Furthermore, with respect to seasonality, it was documented the cover winter crops along agricultural landscapes provide additional food resources for natural predators, thus enhancing their effectives, fitness and the overall pest control service they provide (Boweres et al., 2021). Aside from harbouring functionally distinct communities, natural and seminatural habitat patches were documented to also support other invertebrate taxa (Snyder, 2019). An analysis of how these types of habitats should be used requires more research in order to gain further improvement. Aside from landscape structure, the functionality of natural enemies is also influenced by the agricultural practices carried at patch scale influence. Predatory activity in agricultural landscapes with tree lines was recorded to decrease in conventional farming patches. In opposition, this activity increased in organic farming patches, underlining once again the importance of the local and large scales interactions (Bonoit et al., 2020).
Functional diversity of carabids was found to be sensitive to various factors throughout seasons, at the landscape level. Reducing the use of pesticide, avoiding bare soils all along the year, keeping pesticide use intensity below the identified thresholds and adopting a wide diversity of tillage strategies at the landscape scale should all contribute to enhance in-field carabid occurrences and richness (Muneret et al., 2002). Additionally, the functional redundancy of spiders and ground beetle communities was documented to increase in response to a reduction in agricultural practices intensity and to an increase in natural and seminatural habitats availability at landscape level (Rusch et al., 2014). Interestingly enough, in large scale experiments, functional diversity of predators was not sufficient to explain the overall pest control, thus further reinforcing the need for concomitant multiscale approaches in the research of natural enemies. (Alhadidi et al., 2019). Future research should focus on the influence that different habitat combinations across landscape exert on balancing crop productivity and ecosystem services provided by useful taxa. Furthermore, in the context of the environmental changes taking place, more research should assess the contribution of agricultural landscape in large scale ecosystem processes, by analysing their insect communities and on how these communities increase the ecosystem resilience against different pressures, such as pest invasion and others.

CONCLUSIONS

Most of the research focused predominantly on three major functional traits (body size, dispersal capacity and feeding preference) and to a lesser degree on functional diversity. Addressing the effect exerted by other traits is important for developing our understanding of natural pest control efficiency.
Most of the research was carried out in Europe and developed countries. In continuing this trend, it is also highly important to address the effect of local and landscape changes on the communities of natural enemies from other geographic zones, where agricultural impact is steeply rising.
The functional traits and functional diversity of natural enemies seems to be shaped by multiple interacting variables from different scales. Furthermore, the responses are usually taxa specific and depend on the characteristics of the habitats and landscapes analysed. Further research should account for these ideas, in order to help develop our knowledge on the inner working of pest control provided by natural enemies and to help preserve this valuable communities and ecosystem service.

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COMMERCIAL AND NATURAL DOG AND CAT FOOD: STUDYING THE BENEFITS AND INCONVENIENCES OF USING CURRENT TYPES OF FEED - A REVIEW

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Abstract

The essential aspects in choosing pet food are the quality and nutrient content of the food. This becomes difficult for pet owners to manage when many diets or types of dogs and cat food are available on the market as will be mentioned in the study: commercial pet food, BARF (Biologically Appropriate Raw Food), home-made or vegetarian diets. There are also many brands and recipes in the commercial pet food industry, which again causes confusion and misleads pet owners. Pet owners now treat their dogs and cats as family members, which is why studies show that globally owners have become more aware of their pets' needs. The current study attempts to present the benefits and drawbacks/disadvantages of different varieties of dog and cat food. By providing an overview of pet feeding patterns, the study seeks to clarify the nutritional needs and highlight the physiological digestive capabilities of two specialized carnivores: the dog and the cat.

Key words: *BARF, carnivores, cat, dog, home-made, pet food.*

INTRODUCTION

The food offered to pets, dogs and cats, can be found in the following forms: commercial pet-food (dry, semi-moist, moist), homemade by the owner from natural ingredients/foods, raw food such as the BARF diet (Biologically Appropriate Raw Food), vegetarian food, and mixed food which is composed of two or more of the food variants already presented.

The preparation of commercial dog food dates back to 1861, and its founder is James Spratt who introduced a product in the form of "biscuits" for dogs to the American market (Fraser-Miller S. et al., 2021)

Commercial dog food is designed specifically for dogs and cats and is now manufactured in a variety of forms: dry or kibble, canned moist food and semi-moist food. Nutrition research centers have also created specialized feeds called "prescription diets" designed to support or enhance the biological functions of the animal's body (FDA, 2016).

Vegetarian dog and cat food can be found either as dry or wet commercial food or as cooked food, prepared by the owner from cooked or raw vegetables, fruit or cereals.

The trend for owners to offer a vegetarian-type food to their pets is ongoing and growing in recent years. Studies estimate a global vegetarian food market value of $ 8.7 billion in 2020 and a future increase in this value (The Insight Partners, 2021).

MATERIALS AND METHODS

The current study, a review, was focused on presenting and discussing the types of dog and cat food currently available on the market and the benefits and drawbacks of their use in the daily diet of pets. The search strategy for the information on types of pet food on the international market was by keywords such as "commercial dog/cat food", "BARF food", "diet", and "vegetarian dog/cat food". In the search for statistical data on the number of dogs and cats worldwide, studies and research conducted between the years 2013-2014, and 2020 for cats, were consulted. Also an annual report conducted in 2021 was used to estimate dog and cat populations from Europe, using the official website of FEDIAF (The European Pet Food Industry).

For the definition of terms such as "prescribed diet" the official FDA website was consulted, and for the provision of current statistics and future trends in the purchase of vegetarian pet food the Insight Partners data platform was accessed.
The data summarised in Table 1 on the benefits and disadvantages of each type of pet food for dogs and cats were selected from studies in the scientific literature and nutrition books for dogs and cats, research conducted between 1994 and 2023. Also for raw feeding and mainly for “Biologically appropriate raw feeding”, the BARF guidelines of 2001 were consulted, as well as studies and questionnaires carried out in 2013 and 2017 on feeding practices used by owners for their pets.

RESULTS AND DISCUSSIONS

Globally the population of both owned and unowned dogs is estimated to be between 700 and 1 billion **(**Hughes et al., 2013, Gompper, 2014) according to data provided by several organizations. Recent studies also estimate (but cannot provide an official number) a population of 600,000,000 free-roaming and owner-owned cats (Kays, 2020).
The European Pet Food Industry provides more concrete information and data on the number of pets in Europe and, subsequently in the European Union, that are in the care of an owner as well as an approximate number that are either in shelters or on the streets.
According to FEDIAF's annual reports, most recently in 2021, there are approximately 92.9 million dogs and 113.5 million cats, with 24% of households in Europe owning at least one dog and 26% owning at least one cat, with the remaining households having an exotic pet such as cage birds, fish, rabbits, turtles or other animals.
With the increase in the number of pets and especially in dog and cat populations, the requirements of these animals and their owners have also become increasingly obvious and even challenging for the commercial pet food industry.
Originally the dog and cat are considered two specialized carnivores, being ancestors of the wolf in the case of the dog or the wild cat - *Felis sylvestris lybica* from North Africa in the case of the domestic cat (Serpell A., 2014).
Some researchers consider the cat an even more specialized predator than the wolf in the study The domestication and history of the cat, researchers classify cats as obligate or strict carnivorous species, although they have the ability to digest some carbohydrates as well (Serpell, 2014).
Carnivores naturally evolved based on the nutrient content of their prey, for example, pre-formed vitamin A (which is synthesized in the prey animal from carotenoids). Elevated ammonia levels generated by the proteolysis phenomenon in carnivores increase urea cycle activity and hence the requirement for arginine (Hynd, 2019).
Taurine is a sulphur-containing amino acid synthesized in noncarnivores from L-cysteine, but taurine cannot be formed in carnivores because they lack the enzyme cysteine sulphate decarboxylase, therefore in cat food guidelines this amino acid is integrated and considered essential (Hynd, 2019). Cats also require high levels of dietary niacin because they have limited ability to convert the essential amino acid tryptophan to niacin (Hynd, 2019).
A study that investigated the level of taurine inclusion in pet food, and how this level is altered by subjecting the food to a thermal process, states that the level of taurine in plants cannot be quantified but significant amounts of this sulfonated amino acid have been found in most animal tissues and especially in muscle (Spitze,2003).
More recent studies also confirm that taurine, the omega-3 fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) as well as vitamin A are predominantly, if not exclusively, found in animal tissues (Dawczynski, 2007).
Current studies discuss not only the need for taurine in cats but also in some breeds of dogs, breeds prone to heart disease, and the lack of this amino acid in food can create serious problems such as dilated cardiomyopathy, according to a study of 24 Golden Retriever dogs. Twenty-three of these dogs were fed commercial diets such as dried food rich in legumes and grain-free commercial dried food, which did not pass the quality tests required by AAFCO.

Therefore following blood tests, taurine levels found in the blood, echocardiography, and nutritional history verified, it was concluded that 23 of the 24 dogs tested suffered from dilated cardiomyopathy secondary to a taurine deficiency in the diet and that dietary taurine supplementation resulted in improved health in the dogs studied (Kaplan et al., 2018).

Furthermore, the literature suggests a genetically transmitted taurine deficiency, which occurs as a predisposition only in certain breeds such as Rottweiler (Petric & Tomsic 2008), Great Dane, Doberman pinscher or other breeds (Stern et al., 2018).

Taking into account the previous discussions and information, in the following, the types of food and their advantages and disadvantages, mentioned by the literature and nutrition research institutes, have been presented.

Commercial pet food for dogs and cats (the most numerous pets in the population) has been designed to facilitate or ease the feeding of animals in order to provide a complete and balanced diet for the pets.

This is why the commercial feed is considered "satisfactory" from a nutritional point of view once it meets certain standards such as complete, balanced, palatable, easily digestible, and safe, and passes the quality tests of the nutritional research organizations - NRC, AAFCO, FEDIAF, but is also optimized in relation to the specific nutritional needs - species, breed, age, physiological state (endogenous factors related to the animal).

Achieving adequate nutritional health involves more than just meeting the nutrient profiles, additional factors need to be considered. An iterative process, in which each factor affecting the animal's nutritional status is assessed and reassessed as often as necessary (Thatcher, 2010), results in a comprehensive nutritional assessment of the patient.

The factors to be evaluated include the animal, diet, feeding management, and environmental factors. Factors specific to the animal include age, physiological condition, the physical activity the animal is subjected to (Thatcher, 2010) but also ailments that occur during the lifetime of the animal.

Pet food is available in three basic forms: dry, semi-wet, and wet. As the names of the categories suggest, the water content differs significantly between the three forms.

Dry dog and cat food according to FEDIAF, has a moisture content of less than 14% and is composed of carbohydrates such as polysaccharides, oligosaccharides, monosaccharides, and fiber; proteins such as animal and vegetable proteins; amino acids; lipids such as vegetable oils and animal fats; vitamins; minerals; and preservatives (Crane S., 2010). Processed dry foods have been produced with a caloric density of 2.7 to 7.1 kcal ME/g food (Hand M., 2010).

According to official sources, semi-moist pet food has a moisture content of between 14% and 60%, and wet food has a moisture content of at least 60%. Studies draw attention to the increased palatability of semi-wet or wet foods such as cans and pouches which can cause dogs to consume this type of food more quickly (Arraujo, 2004).

Dry feed is produced by the extrusion process (Elsey, 1997), a process that uses high steam pressure and heat by passing ingredient mixtures through a mould (a machine equipped with a spiral) (Pop, 2006), resulting in compound feeds for pets.

The types of commercial pet food are appreciated by owners both economically and in terms of packaging because they are easy to store and have a long shelf life, these are just some of the advantages found about this type of food, the others have been presented in Table 1.

Figure 1 illustrates the types of food most commonly available on the market and most commonly used by dog and cat owners. In the figure shown, connections have also been made between certain types of food such as home-cooked food and raw food and vegetarian food which can be seen as both commercial food and home-cooked food:

Table 1. Benefits and drawbacks of the types of food currently used in dog and cat nutrition

Dry/semi-wet/wet food	Home-made food	Raw food	Vegetarian food
Benefits - according to the manufacturer the food contains all the nutrients the dog or cat needs[(1), (2)]; - the food is conveniently packaged and easy to store and has a long shelf life[(1), (2)]; - are convenient types of food for owners being the easiest way to feed their companion[(1)]; - feed formulas are available for different life stages, breeds, or medical conditions[(1)]; - feeding guidelines for the weight of dogs or cats also appear on packaging.	Benefits - feeding the animal a natural diet cooked from food chosen by the owner[(3)]; - a diversified diet[(3)]; - no additives, preservatives or palatants are added[(3)]; - the desire of dog and cat owners to improve the relationship between them and their pets[(13)].	Benefits - digestive enzymes in fresh foods increase biological availability[(6)] - a positive effect on the immune response[(7)] - reduction of dental plaque deposition[(7)] - increased physical activity and better general condition of the animal[(7)]	Benefits - a low risk of cardiovascular disease (10) - improved skin and coat appearance (11); - reduction of signs of arthritis (11); - a low risk of obesity (11);
Drawbacks - most products are multifunctional (formulated for all breeds, sometimes for all ages)[(2)]; - products sold in bulk, without package and specifications may have nutrients above the maintenance level of a sedentary dog or cat and may have a relatively low level of nutrients for the dog used to intense activities[(2)]; - labels and ingredients in pet food cause confusion among owners[(3)].	Drawbacks - 17% of pet owners mention the internet as their main source of food recipes[(4)]; - some diets are not balanced and not nutritionally adequate to support adult maintenance[(3)] - calcium/phosphorus ratio and vitamins A and E were below current nutrient recommendations[(5)].	Drawbacks - a high risk of microbial contamination in both commercial and home-prepared BARF diets; - public health risk[(8)]; - environmental contamination is also possible as a result of the excretion of pathogenic microorganisms[(9)].	Drawbacks - vitamin B12 deficiency (10); - lower levels of iron (10); - significantly lower folic acid (10); - urine alkalinization (12). - changes in blood biochemical parameters that may indicate muscle destruction that was associated with cardiomyopathy in cats(14).

Sources: [(1)]FEDIAF, 2021; [(2)]Girginov D., 2007; [(3)] Schenck P., 2010; [(4)] Laflamme D., 2008; [(5)] Diquelou A., 2005; [(6)]Prochaska L.J., 1994; [(7)] Morgan et al.., 2017; [(8)] Lejeune J., 2001; [(9)]Finley R., 2006; [(10)]Schlesinger D., 2011; [(11)] Peden J.,1999; [(12)] Knight A. et al., 2016; [(13)] Pedrinelli V. et al., 2017; [(14)] Dominguez-Oliva et al., 2023.

- home-cooked food can, depending on the wishes of the dog or cat owner, be prepared in such a way that the ingredients added to the food are either thermally prepared or not.;
- raw food, which is not thermally prepared, can be prepared from ingredients or sources of animal origin such as raw meat, raw bones, raw eggs; or vegetarian and contain only sources of plant origin such as vegetables, fruit, etc.

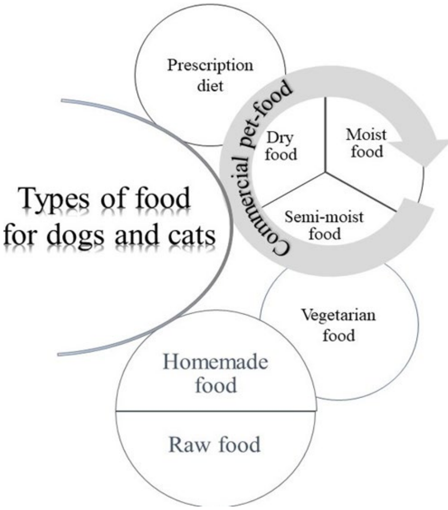

Figure 1. Types of food for dogs and cats

The BARF diet, originally called 'bones and raw food' and later 'Biologically appropriate raw food' created by Australian veterinarian Ian Billinghurst in the 1990s, is also integrated into the raw food diet as a 'natural' alternative to commercially processed food.
The creator of the BARF diet, Billinghurst considered dogs - like cats - obligate carnivores, so he stated that adding carbohydrate sources to a dog's food is unhealthy and therefore not recommended.
In recent years, the tendency to feed dogs and cats a BARF (biologically appropriate raw food) diet has become increasingly popular. It was estimated that the number of pet owners feeding their dogs all or part of a raw diet meets in some European countries is up to 51% (Corbee, 2013).
Among the disadvantages of the consumption and use of raw food, researchers also point out the risk for public health, due to the use of the BARF diet, for owners and other household members who are exposed daily to the transmission of pathogenic microorganisms from raw meat (Lejeune, 2001).
Environmental contamination is also possible as a result of the excretion of pathogenic organisms from the definitive host, the dog or cat, which may be asymptomatic carriers (Finley, 2006).
Home-cooked food should be prepared according to the recipe given by the nutritionist, veterinarian or found in books, without substituting, adding, or omitting an ingredient as each ingredient in the diet is essential, providing the level of specific nutrients needed, therefore correct preparation of home-cooked diets requires time and effort.
Home-made diets, according to specialists, do not contain preservatives and have a high moisture content, thus they become susceptible to fungal and bacterial contamination if left at room temperature for more than a few hours and not stored as directed at refrigeration temperatures (Schenck, 2010).
Similar to the practice of vegetarianism in humans, the vegetarian diet for pets involves the exclusion of animal protein sources or any animal products or by-products from the diet of dogs and cats (Matthew, 2012).
This shift in dogs' diets towards a vegetarian diet sometimes comes in response to the inability to feed the dog animal protein due to the onset of atopic dermatitis, say some pet owners, an increasingly common skin disease in some breeds of dogs like Labrador Retrievers, German Sheperds, Pit bulls, Pugs, Boxers, Shih tzus and others (Miller, 2013).
Some researchers believe that a diet based on raw fruits and vegetables lowers the risk of heart disease based on improved LDL cholesterol and triglyceride concentrations, but note that there is a possible increased risk of coronary heart disease due to cyanocobalamin (vitamin B12) deficiency (associated with increased plasma homocysteine and lower concentrations of HDL - lipoprotein synthesized and secreted in the liver) (Schlesinger, 2011).
A current study conducted in 2023 states that in cats fed a vegetarian diet changes in blood biochemical parameters were observed, specifically creatine kinase activity was increased and may indicate muscle destruction that was associated with cardiomyopathy, but

the study does not indicate the same changes in dogs (Dominguez-Oliva et al., 2023).
The humanization of the pet has also had an impact on the diversity of food types, as could be observed throughout the study, with the culinary tendencies adopted by humans often being imposed or tried on dogs and cats.

CONCLUSIONS

Choosing the right type of food or diet for a pet dog or cat has become an elusive goal with so much on offer.
This study attempts to present and explain the types of food available on the market and to provide information from scientific research, suggesting that the correct diet for dogs and cats would be one that provides nutrient sources that meet the specific nutritional needs of carnivores, the descendants of wolves and wild cats, the obligate carnivores.

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RESEARCH REGARDING THE INFLUENCE OF ORGANIC SELENIUM ON THE IMUNE RESPONSE IN SWINE

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Abstract

Currently, immunomodulation is an alternative in the fight against many diseases, being considered a possibility to fight against many infectious diseases that can affect pig herds. Selenium can be used for this purpose, the effect of its administration on the immune response being the main purpose of the present study. Following the administration of organic selenium, we found that in the case of WBC parameters, granulocytes and agranulocytes percentages and lymphoblastic transformation percentages of B lymphocytes, there are no major differences between the values recorded at the beginning of the experiment and the values recorded in the two experimental moments. Instead, following the administration of organic selenium, we observed significant increases in T lymphocytes percentages (by 9.94%, after 21 days, and respectively by 8.18%, after 30 days), percentages of lymphoblastic transformation of T lymphocytes (by 59.59% after 21 days, and respectively by 64.14% after 30 days), as well as the helper T lymphocytes/ supressor T lymphocytes ratio (by 46.15% after 21 days, and respectively by 65.38% after 30 days). Regarding the percentage of B lymphocytes, a decrease of this parameter is observed by 38.34% after 21 days and by 21.84% after 30 days following the administration of the product based on organic selenium.

***Key words**: immunomodulation, lymphocytes, pigs, selenium.*

INTRODUCTION

Currently, immunomodulation represents an important alternative in combating numerous diseases of pets as well as of economic interest animals, especially in the perspective of banning the use of antibiotics. The importance of the application of immunomodulatory therapies exerts influence on both animal health (Byrne et al., 2014) and public health according to the common desire formulated on the basis of the "One Health" principles (Savu & Petcu, 2002; Petcu, 2006; Petcu et al., 2007).
The use of immunomodulatory preparations in pig herds, both in the case of professional holdings and in the case of households, can increase the subjects' resistance to the action of pathogens, by positively regulating the duration and intensity of the immune response (Gâjâilă et al., 2022). Through the development and application of vaccines throughout history, numerous biological threats have been defeated, but techniques to enhance the non-specific immune response have not benefited the same attention in the research field. Non-specific immunomodulation becomes fundamental alongside the principles of biosecurity in the case of the evolution of infectious pathologies, that, as of now, have no discovered vaccines.
Immunomodulation is still an area of interest for the veterinary medicine research field, being considered a potential weapon in the fight against pathological entities which produce major economic losses in pig herds. If the immunosuppressive therapies address those situations in which it is necessary to suppress a harmful immune response, immune stimulation may represent a prophylactic or therapeutic alternative in case of infectious or parasitic pathologies.
It is known that the use of antibiotics in animal feed can have an immune-stimulating role, but

the legislation of the European Community prohibits them, as factors for preventing antibiotic residues in food products of animal origin (Gallois et al., 2009; Pogurschi et al., 2015; Ghimpețeanu et al., 2022; Pogurschi et al., 2022). Nowadays, the administration of mineral supplements is a common practice in animal husbandry (Ghiță et al., 2021), some of which have an immune-stimulating effect.

From the category of essential minerals for supporting the immune system in pigs, the most cited in the specialized literature are iron and selenium (Marin et al., 2013; Zhou et al., 2021; Falk et al., 2019).

Selenium plays a very important role in the protection of cell membranes against oxidative stress. Numerous selenium-dependent enzyme structures constitute elements of the antioxidant mechanisms so important in the organism defense against pathogens. Organic selenium or inorganic selenium is used as a supplement for animal forage. The inorganic form of selenium, namely selenite or selenate, presents a number of disadvantages compared to organic selenium. Interactions with other minerals in the body, a higher toxicity and a lower storage capacity in the form of reserves are more often discussed in its case. Thus, the organic form of selenomethionine obtained from natural yeast cultures is often preferred in both humans and animals due to its superior bioavailability. Organic forms of selenium are absorbed with an efficiency of about 70-90% while the absorption of selenite does not exceed 60%. Of particular importance is the fact that selenium, among all the elements, has very close values between the threshold characteristic of this mineral's deficiency (<40 µg/day) and the toxic level (>400 µg/day).

The goal of our research was to evaluate by modern techniques the non-specific immune response, in the case of pigs administered organic selenium.

MATERIALS AND METHODS

In the present study, we verified the effectiveness of a commercial preparation based on organic selenium (Sel-Plex®), considered to have an immunomodulatory effect.

Sel-Plex®, additive approved at European level, represents a genuine source of organic selenium, through its composition based on the presence of yeast (*Saccharomyces cerevisiae*). It was administered to pigs by inclusion in the feed in accordance with the manufacturer's recommendations (100 mg/kg feed). At this concentration, the animals in the experimental group did not have a daily intake of selenium higher than 0.5 mg/kg body weight.

In order to pursue the objectives of the present study, a homogeneous batch of 20 pigs of similar ages (4-5 months) belonging to a farm in the area of Cernica, Ilfov county, was established. In choosing the subjects in the study, it was ensured that the individuals were as homogeneous as possible in terms of genetic characteristics, age difference, maintenance conditions and fattening status, considering the influence of some hormones secreted by adipose tissue on lymphocyte populations (Ghiță et al., 2021).

The samples were collected as followed: the first series of samples, prior to the administration of the organic selenium-based product, the subsequent samplings taking place after 21 and 30 days respectively from the time of inclusion of the Sel-Plex® product in the feed. We mention that throughout the administration of selenium, the animals were not treated with any medicinal products, taking in consideration their effect (especially anti-inflammatory drugs) on leukocytes and implicitly on the immune response (Codreanu, 2018; Ghiță et al., 2015).

The immune status evaluation and the implications of the administration of the product based on organic selenium in the tested pigs batch was carried out by classical haematological and immunological techniques with high applicability in veterinary laboratories.

The chosen haematological analysis consisted in the determination of WBC and granulocytes/agranulocytes ratio using an IDEXX type analyzer. The cellular immunology tests included the separation of the total population of lymphocytes in a density gradient with Ficoll medium, the determination of the percentage of T and B lymphocytes by the EA rosette technique and the establishment of the existing ratio between the subpopulations of helper T lymphocytes and suppressor T lymphocytes by the E rosette technique. To obtain pure populations of T and B lymphocytes, the technique of separation by adhesion to nylon

fibers was used, and the evaluation of the functionality of these cells was performed by the lymphoblastic transformation test (TLT), the variant based on the determination of the glucose consumption index in the reaction medium.
The mitogen used in the test was phyto-hemagglutinin (PHA). To test the phagocytic functions, the population of polymorphonuclear neutrophils separated from the blood with the Dextran medium was chosen for determining the locomotion capacity in a Boyden type filter layer with the evaluation of the migration density.
The statistical analysis was performed with the help of the t-test (Student test).

RESULTS AND DISCUSSIONS

The obtained results will be presented in the form of a centralizing table and some graphs, accompanied by short comments. Table 1 presents analysed parameters for the two experimental moments.

Table 1. The values obtained for the experimental moments

Analysed parameters	Before selenium administration	21 days after selenium administration	30 days after selenium administration
Leukocytes totale (10^9/l)	**15.1**	**14.9**	**15.9**
Granulocytes, %	**41**	**43**	**41**
Agranulocytes, %	**59**	**57**	**59**
T lymphocytes, %	**79.4**	**87.3***	**85.9***
B lymphocytes, %	**20.6**	**12.7***	**16.1***
hTL/sTL ratio	**2.6**	**3.8***	**4.3***
Lymphoblastic transformation TL, %	**39.6**	**63.2***	**65.0***
Lymphoblastic transformation BL, %	**41.1**	**39.2**	**42.4**
Directed migration density (μ)	**1702**	**2140***	**2215***

*$p<0.01$

Studying the data presented in Table 1, it can be observed that in the case of WBC parameters, granulocytes and agranulocytes percentages and lymphoblastic transformation of B lymphocytes percentage, there are no major differences between the values recorded at the beginning of the experiment and the values recorded in the two experimental moments. For the other parameters analysed, it can be observed that their values undergo changes in the case of the two experimental moments, changes that will be presented below.
Following the administration of the Sel-Plex® product, there is an increase in % lymphocytes T by 9.94%, after 21 days, and respectively by 8.18%, after 30 days (Figure 1), in both cases the differences being significant from the point of view statistically ($p<0.01$).

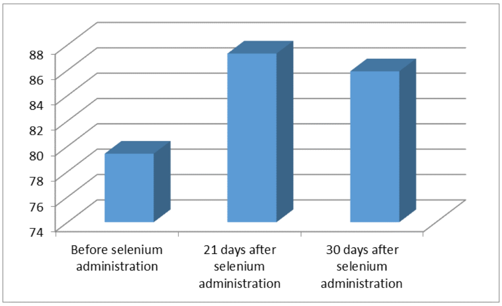

Figure 1. T lymphocytes % during the experiment

Regarding the B lymphocytes percentage, there is a decrease of this parameter by 38.34% after 21 days and by 21.84% after 30 days (Figure 2), in both situations the differences are statistically significant ($p<0.01$).

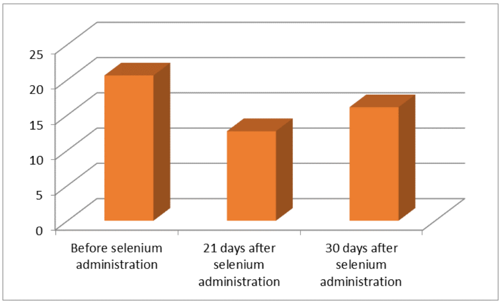

Figure 2. B lymphocytes % during the experiment

The indicated change can be explained by the accelerated proliferation induced by the administration of the Sel-Plex® product, of the population of T Lymphocytes, cells responsible for cell-mediated immunity. Therefore, clones of activated T lymphocytes become dominant and the population of B lymphocytes suffers a numerical decline against the background of granulocytes/agranulocytes ratio maintained constant. Such changes in the ratios between the different types of leukocytes are also found in the case of the administration of other products for immunostimulatory purposes (Li et al., 2020; Ioniță et al., 2014).
Regarding the helper TL/suppressor TL ratio, it was found to increase by 46.15% after 21 days, and respectively by 65.38% after 30 days following the administration of the Sel-Plex product (Figure 3), in both cases the differences being significant ($p<0.01$).

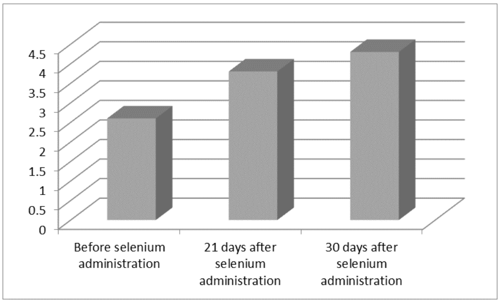

Figure 3. Helper TL/suppressor TL ratio during the experiment

The helper TL/suppressor TL ratio represents the state of balance necessary for the regulatory T lymphocyte subpopulations in order to carry out the immune response mechanisms. The resulting parameters indicate a significant increase in the subpopulation of T helper lymphocytes, which demonstrates that through their functions these cells non-specifically activate a series of effector cells of cellular type immunity (Gâjâilă et al., 2015). The decrease in the proportion of T lymphocytes in this report doesn't reach such values that indicate a state of harmful immunodepression, but a reactivation of some cytotoxic and phagocytic functions independent of the presence of specific antibodies. Regarding the lymphoblastic transformation of TL percentage, it is observed that it increased by 59.59% after 21 days, and respectively by 64.14% after 30 days following the administration of organic selenium (Figure 4), in both cases the differences being significant from a statistical point of view ($p<0.01$).

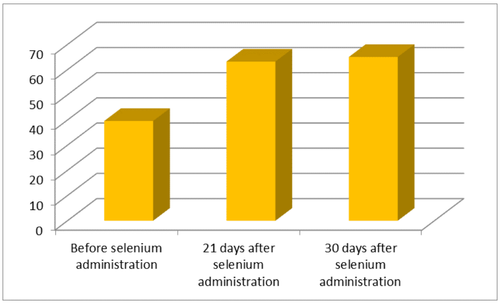

Figure 4. TL lymphoblastic transformation % during the experiment

The lymphoblastic transformation test clearly indicates the reactivity of lymphocyte populations to non-specific activating factors such as selenium. T and B lymphocytes stimulated with selenium and plant mitogens proved to be much more reactive in the case of T lymphocytes, where the transformation indices almost doubled. It is possible that administered selenium acts mainly on T lymphocytes and very little on B lymphocytes, a fact also reported by other authors (Vetvicka et al., 2014; Zavodnik et al., 2011).
In the case of directed migration density, it is observed that this parameter increased by 25.73% after 21 days, and respectively by 30.14% after 30 days from the initiation of the treatment with organic selenium (Figure 5), in both cases the differences being significant from a statistic point of view ($p<0.01$).

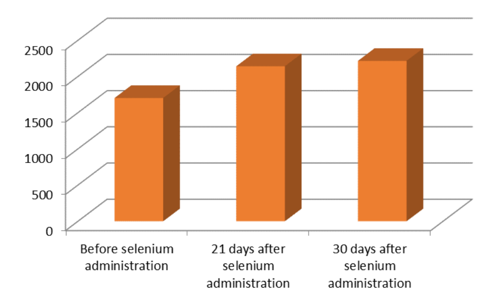

Figure 5. Directed migration density during the experiment

The phagocytic function performed by the population of polymorphonuclear neutrophils is conditioned by the perception of the chemotactic stimulus that triggers the stages of the phagocytosis mechanism (Thacker, 2010). Directed migration demonstrates the activation of the phagocytic cell and constitutes the first step in the evaluation of this function. The results indicate an increase in neutrophil reactivity after the administration of the selenium-based product, an aspect reported in the specialized literature (Dalgaard et al., 2018; Gâjâilă et al., 2016).
Following the administration of the Sel-Plex® product, the dominant changes appear in the results obtained in the evaluation of the HhTL/sTL ratio, the index of lymphoblastic transformation in the case of B lymphocytes and the locomotion capacity by applying the phagocyte migration technique under an agarose layer. These results indicate a specific activation of the cell-mediated immune response in the direction of the coordination functions of the subpopulation of hT lymphocytes and the phagocytic function of neutrophils.
The establishment of the values of the T and B lymphocyte populations, together with the evaluation of the ratios between the 2 categories of cells, showed that the Sel-Plex® product has the potential to increase the proportion of T lymphocytes. The orientation of the TL/BL ratio towards high values of 4, with a weight higher than the 80% value of T lymphocytes, can demonstrate an activation of the proliferation process of T lymphocytes and implicitly the switching of the body's defense functions towards a stronger cell-mediated immune response. Evaluating the reaction to the chemotactic stimulus in the Boyden filter and the locomotion capacity of the circulating neutrophils demonstrated an increase in chemotaxis manifested by an increased migration of neutrophils through the filtering layer. This effect is translated by the optical density values for the directed migration which increased on average by 30%. Similar values were also recorded in the evaluation carried out after 30 days of product administration.

CONCLUSIONS

The administration of selenium in the form of Sel-Plex®, product with an immunomodulatory effect, did not produce major changes in the leukogram values, the ratio of granulocytes and agranulocytes remaining constant in the reference range characteristic of the species. Major changes were found within the subpopulation of lymphocytes.
A change in the TL/BL ratio was identified in the sense of increasing the subpopulation of T lymphocytes without exceeding the maximum threshold considered normal for the development of an effective immune response. The reaction denotes an activation of cellular immune response mechanisms.
The administration of the Sel-Plex® product has identified implications in the coordination mechanisms of immune processes, organic selenium increasing the helper TL/suppressor TL ratio in favor of helper T lymphocytes, to significant values that don't exceed the maximum physiological threshold allowed in pigs, which eliminates possible hypersensitivity states.
The functional evaluation of T and B lymphocytes carried out by the lymphoblastic transformation test demonstrated that the T lymphocyte transformation index increased significantly, a characteristic element that indicates a non-specific but significant stimulation of T lymphocyte subpopulations participating in cellular immune response mechanism.
The use of the Sel-Plex® product demonstrated that it has also generated a stimulation of the specific phagocytic function of neutrophils by stimulating a higher locomotion activity correlated with an intensified chemotaxis.
The tests carried out indicate the immunostimulatory potential of selenium-based products in the immune reaction in pigs, but

great attention must be paid to the doses of selenium administered and the time suitable for a real stimulation because the toxic effects induced by selenium are also known to take place.

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USING STINGING NETTLE (*Urtica dioica*) in POULTRY NUTRITION

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Abstract

Stinging nettle (Urtica dioica) which belong to the family Urticaceae in the major group Angiosperms (flowering plants) is a wild, herbaceous, perennial flowering plant. Stinging nettle is considered a weed by intensive agriculture. However, nettle leaves are good sources of protein, fat, carbohydrates, vitamins, minerals and trace elements. Besides, stinging nettle leaves contain a significant number of biologically-active compounds such as terpenoids, carotenoids and fatty acids, as well as various essential amino acids, chlorophyll, vitamins, tannins, sterols, polysaccharides, isolectins and minerals. Stinging nettle has antiproliferative, anti-inflammatory, antioxidant, analgesic, anti-infectious, hypotensive and antiulcer properties, as well as the ability to prevent cardiovascular diseases thanks to the biologically-active compounds it contains. Due to its biological properties, availability and simple processing technology, stinging nettle can be thought of as an excellent nutritional supplement for poultry. The aim of this review is to evaluate the potential of stinging nettle utilization in poultry nutrition in light of the studies conducted.

Key words*: nutrition, pharmacological activities, poultry, stinging nettle, usage possibilities.*

INTRODUCTION

With the rapid growth of global population and living standards, demand for poultry and livestock production has increased rapidly, leading to intensive poultry production with rapidly developing breeds and high stocking density. However, these conditions have made poultry highly susceptible to infectious diseases as a result of a weakened immune system. Weakened immunity can be caused by various factors such as stress, nutrition, and infectious agents. This has become a recurring economic problem in commercial poultry flocks (Sharma et al., 2018; Zhang et al., 2020; Skomorucha & Sosnówka-Czajka, 2021). Antibiotics have been used in poultry rations for years to eliminate these unfavorable conditions, improve growth and feed utilization, and reduce mortality. However, the continuous use of antibiotics in diets has led to cross-resistance in humans and the development of antibiotic-resistant bacteria, resulting in the ban of antibiotics in poultry rations and directing poultry producers to reduce the use of antibiotics and find potential alternatives in poultry rations (Moula et al., 2019). Recently, medical and aromatic plants and their extracts have gained great interest due to their potential for use as growth promoters and for managing and treating various diseases and conditions. Phytopharmaceuticals are not only cheap and affordable but also accepted as safe, non-antibiotic alternatives to antibiotics by consumers, as they cause either no or minimum side effects (Mansoub, 2011a; Sharma et al., 2018; Abdul-Majeed et al., 2021). One of the medicinal plants that has gained attention as a phytogenic feed additive in poultry rations is stinging nettle (*Urtica dioica*), which has a long history of traditional medical use in many countries. Despite its potential, nettle is considered a wild plant in intensive agriculture. However, its biological characteristics, sufficient availability, and simple processing technology make it a good feed supplement in poultry farms (Krawęcka et al., 2021; Milosevic et al., 2021).

Stinging nettle leaves were reported to be excellent nutritional and functional values. The leaves contain are rich in proteins, fats, carbohydrates, vitamins, minerals, and trace elements and also contain many biologically active compounds. This makes the leaves suitable for feeding monogastric animals such as chickens (Bekele et al., 2015; Moula et al., 2019; Zhang et al., 2020; Abdul-Majeed et al., 2021; Devkota et al., 2022).

This review intends to evaluate the suitability of stinging nettle (*Urtica diocia*) as a potential feed ingredient for poultry by examining its nutritional and pharmacological properties, based on previous research conducted on its utilization.

DESCRIPTION OF STINGING NETTLE

Stinging nettle is a weedy perennial plant that belongs to the family Urticaceae and *Urtica* genus. The *Urtica* genus contains around 46 species of flowering plants. The two most well-known species are the stinging nettle (*Urtica dioica*) and the small nettle (*Urtica urens*). The genus name *Urtica* comes from the Latin verb "urere," which means "to burn," referencing the stinging hairs containing a fluid of formic acid and histamine found on the leaves and stems of the plant. The species name "dioica" of the stinging nettle (*Urtica dioica*) means "two houses" referencing the plant usually has separate male and female flowers on separate plants. It can grow up to a height of around 2 to 4 meters. It has characteristic pointed leaves and small greenish-white flowers. Stinging nettle is distributed almost worldwide, but is particularly common in Europe, North America, North Africa and parts of Asia (Ahmed & Parsuraman, 2014; Kregiel et al., 2018; Moula et al., 2019; Petruzzello, 2022). Taxonomy of stinging nettle has been shown in Table 1.

Table 1. Taxonomy of stinging nettle

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta - Vascular plants
Superdivision	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	Magnoliopsida - Dicotyledons
Subclass	Hamamelididae
Order	Urticales
Family	Urticaceae - Nettle family
Genus	*Urtica* L.
Species	*Urtica dioica* L. - stinging nettle

NUTRITIONAL COMPOSITION OF STINGING NETTLE

Stinging nettle is a good source of nutritional components, such as protein with a well-balanced amino acid profile, fat, carbohydrates, vitamins, minerals, and trace elements. However, various factors, such as variety and genotype, climate, soil, vegetative stage, harvest time, storage, processing, and treatment can influence the chemical composition of nettle plants. Rutto et al. (2013) reported that harvested upgrowths in Fall contained 89% moisture, 3.7% protein, 0.6% fat, 2.1% ash, 6.4% dietary fiber, 7.1% carbohydrates and 45.7 kcal/100 g energy, 4935 IU/100g Vitamin A, 1.1 mg/100 g Vitamin C, 278 mg/100 g calcium, 1.2 mg/100 g iron, while harvested upgrowths in Spring contained 75% moisture, 6.3% protein, 1.4% fat, 3.4% ash, 9.7% fiber, 16.5% carbohydrates and 99.7 kcal/100 g energy, 11403 IU/100 g Vitamin A, 0.5 mg/ 100 g Vitamin C, 788 mg/100 g calcium, 3.4 mg/100 g iron. On the other hand, it was reported that nettle leaf powders contain on average 8% moisture, 30% protein, 4% fat, 15% ash, 10% fiber, 40% carbohydrates and 310 kcal/100 g energy, 170 mg/100 g calcium, 230 mg/100 g iron (Rutto et al., 2013; Adhikari et al., 2016; Kregiel et al., 2018). Composition of stinging nettle leaves is summarized in Table 2.

Table 2. Chemical composition of stinging nettle leaf powder (Adhikari et al., 2016)

Moisture, %	7.04
Crude protein, %	33.77
Crude fat, %	3.55
Crude fiber, %	9.08
Total ash, %	16.21
Carbohydrates, %	37.39
Calcium, mg/100 g	168.77
Iron, mg/100 g	227.89
Tannin content, %	0.93
Polyphenols, mg GAE/g, db	128.75
Carotenoids, µg/g, db	3496.67
Energy, kcal/100 g	307.24

GAE: gaelic acid equivalent; db: dry basis

PHYTOCHEMICAL COMPOSITION OF STINGING NETTLE

Various studies on the nettle have revealed the presence of more than fifty different chemical components. Stinging nettle (*Urtica dioica*) contains a wide range of phytochemicals, many of which have potential health benefits, including flavonoids, phenolic acids, amino acids, carotenoids, organic acids, fatty acids, tannins, terpenes, chlorophyll, sterols, isolectins (Joshi et al., 2014; Said et al., 2015; Grauso et al., 2020; Milosevic et al., 2021; Taheri et al., 2022). Some bioactive constituents of stinging nettle were given Table 3.

Table 3. Bioactive constituents of stinging nettle (Devkota et al., 2022)

Chemical Group	Compounds
Flavonoids	Amentoflavone, apiin, apigenin, apigenin 7-O-β-D-glucoside, baicalin, baicalein, catechin, epicatechin, epigallocatechin gallate, chrysoeriol, genestein, isorhamnetin, kaempferol, keampferol 3-O-β-D-glucoside, luteolin, luteolin 7-O-β-D-glucoside, myrecetin, naringenin, quercetin, quercetin 3-O-β-D-glucoside, quercetin 3-O-β-D-galactoside, rutin, vitexin.
Phenolic acids	**Hydroxybenzoic acid derivatives** Gallic acid, vanillic acid, syringic acid, protocatechuic acid, gentisic acid **Cinnamic acid derivatives** Cinnamic acid, caffeic acid, p-coumaric acid, ferulic acid, chlorogenic acid, sinapic acid
Amino acids	Alanine, γ-aminobutyric acid, glutamic acid, isoleucine, leucine, phenylalanine, proline, tyrosine, valine
Carotenoids	β-Carotene, lutein isomers, neoxanthin, violaxanthin
Organic acids	Acetic acid, citric acid, formic acid, malic acid, succinic acid
Fatty acids	Arachidic acid, arachidonic acid, behenic acid, dodecendioic acid, euric acid, palmitic acid, palmitolic acid, stearic acid, tricosanoic acid, lauric acid, etc.

PHARMACOLOGICAL PROPERTIES OF STINGING NETTLE

Stinging nettle has been reported to have various pharmacological activities such as antimicrobial, antioxidant, antidiabetic, anticancer, antiulcer, anti-inflammatory hypocholesterolemic, hepatoprotective activity and immune-modulatory activity (Majedi et al., 2022)

ANTIMICROBIAL ACTIVITY

Stinging nettle (*Urtica dioica*) has the potential to exhibit antibacterial effects due to its significant amounts of hydroxycinnamic acids (such as chlorogenic acid, caffeic acid, and rosmarinic acid) and flavonoid quercetin.
Both ethanol and aqueous extracts of *U. dioica* have demonstrated antibacterial activity against a wide range of bacteria, including both Gram-positive and Gram-negative strains including *Escherichia coli, Salmonella typhi, Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Shigella flexneri, Enterobacter aerogenes, Pseudomonas aeruginosa, Staphylococcus epidermis, methicillin-resistant (MRSA)* and *methicillin-sensitive (MSSA) Staphylococcus aureus* strains and multi drug resistant bacteria - *Mycobacterium semegmatis.*
Also, extract of nettle showed antifungal activity against *Rhizoctonia solani, Fusarium oxysporium, Fusarium solani, Alternaria alternate* and antiviral activity against HIV-1, HIV-2, CMV, RSV, and flu (Joshi et al., 2014; Kregiel et al., 2018; Rajput et al., 2018; Devkota et al., 2022; Teheri et al., 2022).

ANTIOXIDANT ACTIVITY

Free radicals and ROS are unstable molecules that can cause cellular damage and are associated with the development of various diseases. The harmful effects of free radicals and reactive oxygen species (ROS) on cells can be mitigated with the use of antioxidants, which are known to have prophylactic and therapeutic properties. Antioxidants work by neutralizing these harmful molecules, thereby protecting cells from damage.
Nettle is considered a natural source of antioxidants due to the presence of flavonoids and phenolic compounds. Several in vitro and in vivo studies have been conducted to evaluate its antioxidant activity, and the results confirm its potential in scavenging free radicals and reactive oxygen species. The antioxidant properties of nettle make it a promising natural agent for preventing oxidative stress-related cellular damage, which can contribute to the development of various diseases (Joshi et al., 2014; Kregiel et al., 2018; Rajput et al., 2018; Milosevic et al., 2021; Devkota et al., 2022; Jaiswal & Lee, 2022; Teheri et al., 2022).

HEPATOPROTECTIVE ACTIVITY

Due to the liver's importance in metabolism, it is crucial to maintain its health and functionality. Unfortunately, the liver can be damaged by various factors, including exposure to toxic substances like chemicals, alcohol, and viruses, which can produce harmful metabolites like free radicals that cause oxidative stress and liver injury. As a result, liver diseases are common and diverse, and the organ is frequently targeted by toxicants. However, medicinal plants are being studied for their potential as safe and effective treatments for liver diseases. These plants contain bioactive compounds such as natural polyphenols, which possess powerful antioxidant properties that can protect the liver from damage. The maximum hepatoprotective activity of the stinging nettle leaf extract was observed at a dosage of 400 mg/kg, as indicated by a decrease in the levels of serum alanine transaminase (ALT), aspartate amino-transferase (AST), alkaline phosphatase (ALP), total bilirubin, and malonyldehyde (MDA), and an increase in the level of superoxide dismutase (SOD). These results demonstrate the efficacy of the leaf extract in preventing liver damage and promoting healthy liver enzyme levels (Kar et al., 2007; Joshi et al., 2015; Deniz, 2018; Rajput et al., 2018; Teheri et al., 2022).

ANTIDIABETIC ACTIVITY

Urtica dioica leaves have the potential as an antidiabetic agent by reducing blood sugar levels, HbA1C percentage, and insulin resistance through their anti-inflammatory properties, which lead to a decrease in C-reactive protein and TNF-α levels in serum, and their antioxidant properties, which decrease MDA levels and increase GSH levels, SOD, and catalase activities in pancreatic tissues. The antidiabetic effect of nettle is attributed to the presence of various compounds, such as polyphenols, triterpenes, sterols, flavonoids, and lectin. Studies on diabetic rat models have shown that Urtica dioica leaves decrease fasting blood glucose, total cholesterol, and total triglyceride levels while increasing HDL and insulin levels. Furthermore, the hydroalcoholic extract of Urtica dioica leaves may help regenerate β-cells and reduce the severity of diabetes when administered before the induction of hyperglycemia. Urtica dioica leaves also demonstrate effectiveness in reducing blood sugar and glycated hemoglobin levels in STZ-induced diabetes and can mitigate the complications of dexamethasone-induced diabetes (Rajput et al., 2018; Zangeneh et al., 2020; Ziaei et al., 2020; Bhusal et al., 2022; Samakar et al., 2022).

ANTI-INFLAMMATORY ACTIVITY

Scientific research has revealed that *Urtica dioica*, commonly known as stinging nettle, has the ability to reduce the inflammatory response through various mechanisms, resulting in decreased synthesis of lipid mediators and proinflammatory cytokines. One of the key mechanisms is the inhibition of the arachidonic acid cascade enzymes, specifically the cyclo oxygenases COX-1 and COX-2, which block the biosynthesis of prostaglandins and thromboxanes. Additionally, *Urtica dioica* has been found to inhibit the NF-kappa B (Nuclear factor kappa-light-chain-enhancer of activated B cells) system, which plays a role in immune, inflammatory, and antiapoptotic responses, as well as the PAF (Platelet activating factor) system. Several studies have also demonstrated that *Urtica dioica* leaf extracts reduce the release of proinflammatory cytokines, including interleukins IL-2 and IL-1β, interferon γ, and TNF-α (Tumour necrosis factor). Overall, the multiple mechanisms through which *Urtica dioica* can reduce inflammation make it a promising natural remedy for inflammatory conditions (Joshi et al., 2014; Said et al., 2015; Carvalho et al., 2017; Rajput et al., 2018; Dhouibi et al., 2020; Grauso et al., 2020; Majedi et al., 2021; Bhusal et al., 2022; Devkota et al., 2022).

ANTI-HYPERLIPIDEMIC ACTIVITY

Atherogenic dyslipidemia is a medical condition characterized by increased levels of low-density lipoprotein particles (LDL), elevated levels of triglycerides (TG), and decreased levels of high-density lipoprotein particles (HDL), in the bloodstream. This condition increases the risk of developing atherosclerosis. As high levels of serum LDL can lead to cholesterol build up in the arteries, while HDL can remove cholesterol from tissues and transport it to the liver for breakdown (reverse cholesterol transport), thereby exhibiting anti-atherogenic effects. Numerous animal and human studies have shown that nettle extract has potential anti-hyperlipidemic effects by lowering lipid and lipoprotein concentrations in the blood, specifically by reducing LDL and increasing HDL levels. In one study, rats were given a daily supplement of 150mg/kg of aqueous nettle extract for 30 days while consuming either a normal or high-fat diet, resulting in an improved blood lipid profile with decreased total cholesterol levels and reduced LDL/HDL ratios by lowering LDL content and plasma total apolipoprotein B levels. In another study, hypercholesterolemic rats were given the ethanoic extract of nettle at doses of 100 and 300mg/kg, resulting in a significant reduction in total cholesterol and LDL levels (Joshi et al., 2014; Rajput et al., 2018; Samakar et al., 2022).

IMMUNOMODULATORY ACTIVITY

Several studies have demonstrated that *Urtica dioica* agglutinin (UDA), a protein isolated from the roots of the stinging nettle plant, has immunomodulatory effects. These effects have been observed in T cells, macrophages, thymocytes, and the release of TNF-α, a cytokine involved in inflammation and immune system regulation. These studies have provided insights into how UDA can stimulate T cell proliferation, enhance macrophage activity, promote thymocyte differentiation, and inhibit TNF-α release, potentially leading to therapeutic applications in immune-related disorders (Akbay et al., 2003; Joshi et al., 2014; Said et al., 2015; Francišković et al., 2017; Sharma et al., 2018).

ANTIULSER and ANTICANCER ACTIVITY

Stinging nettle (*Urtica dioica*) has been investigated for its potential anti-ulcer and anti-cancer activities. In an animal study, a water extract of stinging nettle was found to reduce mucosal injury by up to 77.8% at a dose of 200 mg/kg, and also decreased stomach acidity in a model of peptic ulcer caused by pylorus ligation. Stinging nettle extracts have also been evaluated for their cytotoxic effects on various cancer cell lines. For instance, the aqueous extract of the plant roots demonstrated a dose-dependent inhibition of cell proliferation in HeLa cells, and reduced viability of MCF-7 breast cancer cells with an IC50 value of 34 µg/ml at 48 hours. In addition, the aqueous extract of stinging nettle leaves was found to have significant anti-cancer activity against LNCaP prostate carcinoma cells, leading to reduced cell viability in a dose-dependent manner. Furthermore, a dichloromethane extract of stinging nettle has demonstrated anti-cancer activity in a mouse model of breast cancer, with reductions in tumor size and weight at doses of 10 and 20 mg/kg b.w/day (i.p.). These effects were attributed to increased cell apoptosis and suppression of cell proliferation through downregulation of BCL2 and increased caspase-3 activity (Gülçin et al., 2004; Burkova et al., 2011; Joshi et al., 2014; Said et al., 2015; Mansoori et al., 2017; Mohammedi et al., 2017; Rajput et al., 2018; Grauso et al., 2020; Ahmed et al., 2022; Taheri et al., 2022).

EFFECTS OF STINGING NETTLE ON POULTRY

Stinging nettle has been regarded as a promising alternative to antibiotic growth promoters due to its various pharmacological effects and natural growth-promoting properties, leading to its investigation by researchers as a potential candidate for use in the poultry industry. Numerous studies have been conducted to examine the impact of stinging nettle, in various forms, on poultry nutrition. In a study investigating the effects of nettle on performance, Mansoub (2011a) reported that the addition of 1.5% nettle to broiler diets increased feed conversion ratio and body weight gain of animals. Similarly, in another study conducted with broilers, it was reported that the addition of nettle in the form of dried leaves at 1-2% level had positive effects on performance, carcass and blood biochemical parameters (Safamehr et al., 2012). Ahmadipour & Khajali (2019), at the end of a trial with broiler chickens using nettle powder, observed that when nettle was added to the diets at 1% and 1.5%, body weight gain and feed conversion ratio improved significantly, breast meat yield increased, and liver, heart, bursa fabricius and abdominal fat ratios were significantly reduced. It was also reported that the addition of nettle can positively affect the fatty acid profile of breast meat by increasing linoleic acid, linolenic acid, and PUFA, while decreasing MUFA, and may improve the quality of breast meat (Stojčić et al., 2016). However, Keshavarz et al. (2014) reported that the addition of 5 and 10g/kg nettle powder to broiler diets had no effect on performance, carcass and blood biochemical parameters. Another study reported that the addition of nettle leaf extract (NLE) powder at 0.15, 0.20, and 0.25% levels to broiler diets improved performance (Hashemi et al., 2018). In the studies conducted with nettle root extract, the addition of 0.05% nettle root extract to the rations increased the live weight and feed conversion ratio of the animals (Tabari et al. 2016; Meimandipour et al., 2017). Apart from the effects of nettle on the performance of poultry, its effects on antimicrobial, antioxidant, antihyperlipidemic, and immunomodulatory activities have also been investigated in various studies. Safamehr et al. (2012) reported that serum triglyceride and cholesterol concentrations in female broiler chicks were significantly decreased under the effect of 1% nettle addition to the diet, but HDL cholesterol was not significant between groups ($P < 0.05$). In another study conducted with male broilers, similar results were obtained with 1.5% nettle supplementation (Mansoub, 2011a). Abdul-Majeed et al. (2021) reported that the addition of 0.5% crushed nettle leaves to broiler diets may improve lipoprotein synthesis and metabolism by increasing HDL levels and decreasing LDL and risk index levels.

In a study conducted by Al-Salihi et al. (2018), the immunomodulatory effect of nettle in broilers was investigated using a water extract of nettle leaves administered through drinking water at three different concentrations (10, 15, 20 ml/l). The results of the study revealed a significant improvement in various immunological traits, including Delayed-Type Hypersensitivity test (DTH), Enzyme-Linked Immunosorbent Assay, relative weight of fabricia bursi and fabricia index, as well as blood constituents such as red blood cells, white blood cells, Packed Cell Volume (PCV), hemoglobin concentration, and heterophil to lymphocyte ratio. These results demonstrate the immunostimulatory potential of nettle leaf extract. Similar to the results of the previous study, Abdul-Majeed et al. (2021) reported that 0.50% chopped leaves increased hemoglobin, PCV, immunoglobulin and white blood cell count in the blood. Hashemi et al. (2018) also reported an increase in haemoglobin and haematocrit blood content in broiler chickens treated with nettle, indicating a potential impact on immune system function. In another study, Şandru et al. (2017) reported that repeated alcoholic nettle extract treatments in broiler chickens improve not only the weight gain but also the non-specific cell-mediated immunity.

The flavonoids and polyphenolic compounds found in nettle exhibit antioxidant properties that help inhibit harmful free radical reactions, affecting positively immunity and general health both directly and indirectly. In a study investigating the antioxidant properties of nettle in broilers, Ahmadipour & Khajali (2019) reported that higher nitric oxide concentration and lower malondialdehyde (MDA), heterophil/lymphocyte ratio (H:L ratio), and hematocrit concentrations were observed with the addition of 1 and 1.5% nettle to broiler diets compared to the control group. Behboodi et al. (2021) reported that the addition of 0.25 ml/l nettle extract to the drinking water of broilers increased total antioxidant capacity (TAC), total superoxide dismutase (SOD), glutathione peroxidase (GPX) and decreased MDA in accordance with the results of previous studies. Mehboob et al. (2022) also reported that the birds in the treatment group given nettle at a 2% concentration had a significantly lower value of Thiobarbituric Acid Reactive Substances (TBARS) compared to the control group. Studies have consistently shown that stress can lead to oxidative damage in the body, which can cause an increase in MDA level. Therefore, the decrease in MDA level as observed in broiler chickens receiving nettle extract indicates the strong antioxidant capacity of nettle. Furthermore, since antioxidant enzymes, including TAC, GPX, and SOD, are responsible for inhibiting free radicals in the body, the increase in their level indicates strong antioxidant capacity. In addition, the HL ratio is a commonly used stress index in chickens and a higher ratio indicates a greater degree of stress. The reduction in the HL ratio observed in nettle-consuming broilers suggests that nettle has a curative effect on oxidative stress in these birds (Xu et al., 2018; Behboodi et al., 2021; Milosevic et al., 2021).

Recently, a study conducted by Skomorucha & Sosnówka-Czajka (2021) investigated the antioxidant capacity of nettle in an induced thermal stress environment. The results of the study revealed that the addition of 2 ml/l nettle extract was effective in reducing the thyroid hormone (triiodothyronine) and rectal temperature in chickens during the initial period of thermal stress.

Moreover, broilers that received nettle extract had lower radiated temperature of the unfeathered body, a lower HL ratio in the blood during the increase in ambient temperature, and the lowest mortality percentage among all the experimental groups. Farahani & Hosseinian (2022) reported that administering 4% stinging nettle powder in the diet of broilers minimized the effects of chronic heat stress by normalizing serum cortisol, total cholesterol (TC), and uric acid (UA) levels. Additionally, this treatment reduced serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK) enzymes, which are indices of tissue damage in broilers exposed to chronic heat stress.

In studies investigating the antimicrobial effect of nettle, it was reported that extracts from the root and leaves of nettle decreased the number of small intestinal coliform and total bacteria, while increasing the number of lactobacillus (Tabari et al., 2016; Al Salhi, 2020). Moreover, several studies have shown that nettle exhibits a protective hepatorenal effect in broilers affected by aflatoxin, while also decreasing certain chemical factors associated with kidney complications, such as nephritis, nephrosis, and urolithiasis (Uyar et al., 2016; Khalesi et al., 2022).

The potential of stinging nettle leaf meal as a viable substitute for soybean meal in broiler diets was also explored. In an experiment where soybean meal was replaced with 3%, 6%, 9%, and 12% nettle leaf meal, no significant difference was observed among the treatment groups. Based on this finding, the authors concluded that incorporating stinging nettle leaf meal in broiler diets up to 9% could be a feasible alternative feeding strategy to soybean meal (Bekele et al., 2015).

There are various studies investigating the effects of stinging nettle not only on broilers, but also on laying hens and quails. Mansoob (2011b) reported an increase in eggshell thickness and yolk index, as well as a decrease in serum total cholesterol, triglyceride, and LDL concentrations with the addition of 2% nettle meal to laying hen diets. A study investigating the effects of 15% nettle supplementation on laying hen feeds found that it increased egg production, eggshell thickness, yolk n-3 polyunsaturated fatty acid levels, and yolk color. Additionally, nettle supplementation decreased both yolk and serum cholesterol content, which is consistent with the findings of a previous study (Zhang et al., 2020). Grigorova et al. (2022) reported that supplementing the laying hen diet with 0.3% and 0.5% nettle had a positive effect on yolk color pigmentation. In addition, 0.3% nettle supplementation resulted in a significant decrease in yolk cholesterol content. Nettle supplementation was also found to significantly reduce blood serum glucose and total serum cholesterol levels. However, the performance of laying hens was not affected by nettle supplementation. However, unlike the findings of previously conducted studies investigating the effects of nettle on laying hens, Kavan et al. (2023) reported that supplementing the diet of laying hens with 100 and 200 mg/kg nettle essential oil did not affect egg quality characteristics or serum parameters. In a study conducted with quails, similar results were obtained to those seen in studies with laying hens. It was reported that supplementing the quail diet with 6% nettle led to a decrease in egg yolk cholesterol, serum total cholesterol, and serum triglyceride concentrations. Additionally, quail performance was not adversely affected by the nettle supplementation (Moula et al., 2019)

CONCLUSIONS

Stinging nettle, typically considered a weed in agriculture, could be a valuable and underexplored resource in poultry production. Recent studies suggest that incorporating nettle into broiler and layer diets can effectively tackle various industry challenges by providing nutrients and bioactive compounds that enhance growth, feed utilization, metabolic regulation, and immune support in poultry. However, more research is needed to fully comprehend the plant's phytochemical composition, mode of action, and nutritional value. In conclusion, stinging nettle has the potential to enhance poultry nutrition and address industry issues, making it a promising and overlooked tool in modern agriculture.

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THE EFFECT OF ALFALFA MEAL USED IN BROILER CHICKEN DIETS ON PRODUCTION PERFORMANCES, EFFICIENCY FACTORS, CARCASS PARTS, ORGANS DEVELOPMENT AND INTESTINAL MICROFLORA

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Abstract

In this study alfalfa meal effects on production performances, efficiency factors, carcass part, organs development and intestinal microflora were tested. For that, 60 Cobb 500 broiler chickens were divided into two groups of 30 broilers/ group and fed a control or experimental diet in the grower and finisher phases. The birds were raised in an experimental room, with controlled microclimate conditions and fed a control diet (C) based on corn, soybean meal and wheat or an experimental diet containing 5% alfalfa meal (A). The production performances showed that the experimental group, containing 5% alfalfa had a significant effect (P<0.05) on feed conversion ratio. Viability rate was higher in the A group, which influenced the European Production Efficiency Factor and the European Broiler Index but without significant effect. At the end of the trial, six broilers from each group were slaughtered, the carcass part and the organs were measured and samples of intestinal and caecal content were collected for microbiological analyses. The thigh muscle, liver and gizzard were significantly (P<0.05) higher in the A group compared with the C group. The effect of alfalfa meal was very efficient in increasing the beneficial bacteria in the intestinal and caecal segment such as Lactobacilli spp., and significantly decreased the total count of harmful bacteria like Staphylococcus spp., Escherichia coli, Clostridium spp., Enterococcus spp., and Coliforms.

***Key words**: alfalfa meal; broiler chickens; feed additive; meat quality; performances*

INTRODUCTION

Alfalfa (*Medicago sativa*) is an important feedstuff for animal feeding being widely used due to its many functional components such as proteins, polysaccharides, saponins, minerals (phosphorus, calcium, potassium, sodium, chlorine, sulphur, magnesium, copper, manganese, iron, cobalt, boron, and molybdenum) and vitamins (A, D, E, K, C, B1, B2, B6, B12, pantothenic and folic acid, inositol, biotin, and niacin). Alfalfa is also rich in xanthophylls and carotenoids and is well-balanced in amino acids (Vlaicu et al., 2021). Alfalfa meal is presenting a broad range of advantages and it is an excellent dietary feed for poultry with different purposes. It has been used in previous research as a feed supplement in laying hens and broiler diets, as a commercially available leaf meal, rich in protein (up to 19% on a dry matter basis) and crude fiber (Jiang et al., 2012; Tufarelli et al., 2018). Although in a recent study, it was reported that the addition of more than 5% alfalfa meal negatively influenced broiler chicken performances (Pliedo et al., 2020), other study reported that 3%, 6%, or 9% alfalfa meal used in broiler diets improved carcass parts (Jiang et al., 2012). All these nutrients can be absorbed by the animals only if the gastrointestinal tract presents a balanced microbial community which further plays an important role in the overall health and function of the host (Shaufi et al., 2015). During the processes of nutrition, metabolism, physiology, and immunity, the gastrointestinal microbiota can promote digestion and absorption of nutrients, stimulate the immune response of the host, and enhance resistance to infection (Zheng et al., 2019). Literature data reported that alfalfa meal can be a good candidate to enhance some carcass edible parts and intestinal health of the host, without negative effect on production performances if added in adequate amounts (Varzaru et al., 2020; Zheng et al., 2019).

The purpose of this experimental study was to test the effect of 5% alfalfa meal on broiler chickens production performances, efficiency factors, carcass parts, organ development and intestinal microflora.

MATERIALS AND METHODS

Ethical Considerations

This study was conducted in the experimental poultry facility located at the National Research and Development Institute for Biology and Animal Nutrition, Romania. All procedures concerning animals' care, handling, and sampling were conducted under the approval of the Ethical Committee of the institute, according to the Romanian legislation (Law 206/2004, ordinance 28/31.08.2011, Law 43/ 11.04.2014, Directive 2010/63/EU) before the initiation of the study and followed the Romanian guidelines.

Animals, Experimental Design and Diets

A total of 60, Cobb 500 broiler chickens were purchased from a local hatchery and randomly distributed into 2 homogeneous groups of 30 chickens each, with 6 repetitions of 5 replicates each. They were housed in an experimental hall equipped with three-tiered Big Dutchman digestibility cages. The temperature inside the experimental hall and the light regimen were set according to the Cobb 500 broiler management guide. For the starter phase (10 days) broilers were fed with the same basal diet. After these 10 days, the chickens were weighed individually and assigned to two groups (C and A) with homogenous weights (483 g/group), and the actual experimental trial started during the grower (11-28 days) and two finisher phases (29-42 days). Corn and soybean meal were used as the main ingredients in the control diet (C) for broilers and the experimental diet was supplemented with 5% alfalfa (A).

The experimental diet was individually prepared by mixing the control diet thoroughly with the 5% of alfalfa meal at the required incorporation levels, as presented in Table 1. Alfalfa was purchased in pelleted form and was milled to the powder and used in compound feeds, which was given to the broiler in mash form. Both diets were composed to meet the requirements suggested by the Cobb 500 Management Breeding Guide being isonitrogenous, iso-energetic, and iso-fibrous. The ingredients and nutritional composition of the diets are shown in Table 1.

Table 1. Ingredients of the control and experimental diets give to the broilers during grower and finishing phases

Ingredients, % as fed-basis	**C**	**A**	**C**	**A**	**C**	**A**
	Grower (11-28 days)		Finisher I (29-35 days)		Finisher II (36-42 days)	
Corn meal	44.94	45.00	45.00	44.10	44.95	44.86
Soybean meal	23.31	24.75	20.30	20.77	16.94	17.33
Corn gluten	5.00	5.00	5.00	5.00	5.00	5.00
Wheat	19.23	11.87	21.79	15.55	24.70	18.51
Sunflower vegetal oil	2.72	4.32	3.52	5.00	3.99	5.46
Alfalfa meal	-	5.00	-	5.00	-	5.00
Acidifying	-	0.10	-	0.10	-	0.10
L-Lysine HCl	0.38	0.10	0.33	0.08	0.36	0.11
DL-Methionine	0.29	0.18	0.26	0.16	0.25	0.14
L-Threonine	0.10	0.10	0.10	0.08	0.08	0.09
Choline	0.04	0.04	0.04	0.04	0.04	0.04
CaCO3	1.32	0.96	1.20	0.85	1.22	0.87
Monocalcium Phosphate	1.31	1.33	1.14	1.15	1.14	1.16
Chlorine	0.36	0.35	0.36	0.35	0.33	0.33
Premix*	1.00	1.00	1.00	1.00	1.00	1.00
Total ingredients, %	100	100	100	100	100	100

Calculated nutrients						
Metabolizable Energy, kcal/kg	3025	3025	3100	3100	3150	3150
Crude Protein, %	19.46	19.71	18.50	18.50	17.50	17.50
Crude fiber, %	3.45	4.43	3.31	4.25	3.15	4.08
Calcium, %	0.84	0.84	0.76	0.76	0.76	0.76
Available Phosphorus, %	0.42	0.42	0.38	0.38	1.05	0.38
Lysine, %	1.21	1.22	1.10	1.11	0.53	1.05
Methionine +Cysteine, %	0.93	0.94	0.88	0.88	0.84	0.84
Threonine, %	0.81	0.81	0.77	0.73	0.70	0.70
Tryptophan, %	0.19	0.19	0.18	0.18	0.17	0.16

The premix contains: 1100000 IU/kg Vit. A; 200000 IU/kg Vit. D3; 2700 IU/kg Vit. E; 300 mg/kg Vit. K; 200 mg/kg Vit. B1; 400 mg/kg Vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg Vit. B6; 4 mg/kg Vit. B7; 100 mg/kg Vit. B9; 1.8 mg/kg Vit. B12; 2000 mg/kg Vit. C; 8000 mg/kg Mn; 8000 mg/kg Fe; 500 mg/kg Cu; 6000 mg/kg Zn; 37 mg/kg Co; 152 mg/kg iodine; 18 mg/kg Se. C- control diet; A - a diet containing 5% alfalfa meal.

Growth Performance, Production Efficiency and Sample Collection

The broiler chickens were individually weighed at 10 days and after the experimental feeding trial started. The weight was recorded (BWi, g), and then on the day of slaughter at 42 days (BWf, g). Body weight was recorded, and based on the differences; the average daily weight gain (ADWG, g/broiler/day) was calculated for the entire experimental period. Average daily feed intake (ADFI, g feed/broiler/day) was registered daily and the feed conversion ratio (FCR, kg feed/kg weight) was calculated. The viability (survival rate, %) of the chickens in each group was monitored during the experimental period.

The European Production Efficiency Factor (EPEF) and the European Broiler Index (EBI) were calculated with appropriate formulas (Vlaicu et al., 2023), based on the results obtained from the production performances.

After the rearing period, at the end of the feeding trial (42 days of age), 6 chickens/group with homogenous weights were selected for slaughter and sampling, following the procedures presented elsewhere (Vlaicu et al., 2020). After that, the defeathered carcass was dissected and eviscerated and the organs were measured. From the intestinal content, the samples from the caecum and small intestine were collected into plastic sterile tubes, placed on ice stored at -20°C until the analyses were performed.

Carcass Edible Parts and Organ Development Measurements

The organs' development was measured during the evisceration process. The weight of the carcass, breast muscle, thigh muscle, gizzard, liver, spleen, bile and full intestine were weighted with a Kern balance with 0.0001 precision. The results are expressed as % of total final body weight.

Intestinal Microflora Analyses

After samples defrost, decimal dilutions in phosphate-buffered saline pH 7.0 (PBS, Dulbecco A; Oxoid Livingstone Ltd., London, England) were performed for enumeration of microbial populations and assessed for analyses of Lactic Acid Bacteria as reported previously (Lefter et al., 2023) after the methods presented by Dumitru et al. (2018).

Statistical Analyses

One-way analysis of variance (ANOVA), using GraphPad Prism, version 9 was carried out to determine the effect of alfalfa meal versus control diet on production performances, efficiency factors and meat intestinal microflora. The significance of individual mean differences was considered at $p<0.05$.

RESULTS AND DISCUSSIONS

The production performances obtained at the end of the experiment are presented in Table 2. Although the average daily feed intake (ADFI) in the group receiving the experimental diet (A) was slightly lower than that of the control group (C), the difference was not statistically significant. This implies that the dietary inclusion of alfalfa did not have a substantial influence on the overall appetite and feed consumption. However, it is noteworthy that the feed conversion ratio (FCR), which measures the efficiency of converting feed into body weight, was significantly higher in experimental group (A) compared to control group C. This indicates that the broilers in

experimental group fed with alfalfa required more feed to produce the same amount of weight gain as the hens in control group, suggesting a reduced feed efficiency in the supplemented group. Additionally, the viability rate in group A was higher that in group C, indicating a potential positive impact of the experimental diet on the broilers survival rate.
Interestingly, despite these observed differences, there were no significant effects on the European production efficiency factor (EPEF) and European broiler index (EBI) between group A and group C. The EPEF and EBI are indices used to assess the overall performance and profitability of poultry production systems. The lack of significant effects it attributed to lower viability rate in the group C in comparison to the experimental group.

Table 2. Production performances and efficiency factors of broiler chickens fed control diet versus alfalfa diet

Item	C	A	SEM	P
BWi, g	483.1	483.5	0.020	0.995
BWf, g	3029	3056	0.055	0.321
ADWG, g	77.66	74.23	0.102	0.102
ADFI, g	150.2	144.1	1.035	1.070
FCR, kg feed/kg BW	1.63[b]	1.75[a]	0.022	0.003
Viability, %	96.20	100	-	-
EPEF, %	468.7	483.9	10.09	0.053
EBI, %	441.8	480.9	22.44	0.235

[a, b] - mean marked with a different superscript letter within each column are significantly different. C - control diet; A - experimental diet with the addition of 5% alfalfa meal; SEM - standard error of the mean; P - significance; BWi - initial body weight; BWf - final body weight; ADWG - average daily weight gain; ADFI - average daily feed intake; EPEF - European production efficiency factor; EBI - European broiler index.

In line with these results, no significant effect (P>0.05) on BW, ADWG or ADFI when alfalfa was used at 3%, 5%, 6%, or 7.5% was also reported in other studies (Jiang et al., 2012; Zheng et al., 2019; Gulizia and Downs 2020; Varzaru et al., 2020). Regarding the increased FCR, when 7.5% alfalfa meal was used in broilers the authors Gulizia and Downs (2020) reported significantly higher FCR from 0 to 21 days of growing and a 5.56% mortality rate. In the study of Varzaru et al. (2020), the FCR increased from 1.91 g/g in the C group, to 2.02 g/g in the experimental group supplemented with 5% alfalfa meal. Contrary, Zheng et al. (2019) declared that 5%, 8% or 10% alfalfa meal used in laying-type chickens decreased (P < 0.05) FCR and mortality rate. Moreover, the utilization of 3%, 6%, and 9% alfalfa meal in growing ducks also did not significantly differ in terms of ADG, ADFI, and feed efficiency from those fed the control diet. No effect on production performances when adding 3%, 6%, and 9% alfalfa meal to laying quails diets was also observed by Güçlu et al. (2004). Based on these findings, it can be concluded that alfalfa meal has a different effect on poultry species, due to a variety of biologically active compounds which act through different pathways and influence differently the production performances and other parameters in poultry. This theory was also recently confirmed by other authors (Cui et al., 2022). Although a lot of contradictory findings are published on the optimal inclusion level of alfalfa in poultry diets, the processing techniques such as pelleting, milling or micronizing are believed to increase the digestibility of protein, starch and apparent metabolizable energy (Tufarelli et al., 2018).
The effect of alfalfa diet compared with the control diet, on carcass, edible parts with commercial interest and organ development is presented in Table 3.

Table 3. Organ development of broiler chickens fed control diet versus alfalfa diet

Item, %	C	A	SEM	P
Carcass	2288	2343	32.55	0.424
Breast muscle	619.4	598.9	23.70	0.105
Thigh muscle	499.7[b]	554.1[a]	12.45	0.019
Liver	60.85[b]	67.82[a]	2.240	0.009
Gizzard	33.90[b]	41.24[a]	1.495	0.005
Heart	15.75	15.85	0.347	0.895
Spleen	2.70	2.71	0.147	0.971
Bile	1.50	1.49	0.135	0.976
Full intestine	164.1	187.7	6.328	0.056

[a, b] - mean marked with a different superscript letter within each column are significantly different. C - control diet; A - experimental diet with the addition of 5% alfalfa meal; SEM - standard error of the mean; P - significance.

The results showed that the thigh muscle, the liver, and the gizzard were significantly (p<0.05) higher in the A group compared with the C group. In line with our results, other authors (Jiang et al., 2012; Zheng et al., 2019; Varzaru et al., 2020) reported that alfalfa meal could improve the carcass traits of poultry. However, when used at 7.5% addition, it was

reported that had no effect on organ development or carcass parts of eviscerated broilers (Gulizia & Downs, 2020). Other authors reported that the early introduction of 5% alfalfa leaves or high alfalfa leaf content (15-20%) significantly decreased performance and carcass weights, due to antinutritional substances such as saponins that naturally occur in alfalfa (Pleger et al., 2020). In contrast to broilers, the ducks fed diets with 3, 6, and 9% alfalfa meal decreased abdominal fat percentage and improved carcase traits, without an adverse effect on performance (Jiang et al., 2012). The hardly controllable dose of phytogenic substances and antinutritional factors could also explain these inconsistencies.
A thorough investigation of the chicken gastrointestinal microbiota is essential to understand their roles in host function, as it is well known that the intestinal microbiota is of great importance to host health and production.
The intestines are populated by a complex and dynamic microbial community, which contributes to the health status of the host animals and is the first barrier against pathogens. In this study, it was found that the alfalfa meal was very efficient in decreasing the harmful bacteria in the intestine and caecum of broiler chickens.
The CFU of *Staphylococcus* spp. (Figure 1) was significantly lower in both intestinal segments (intestine and caecum).

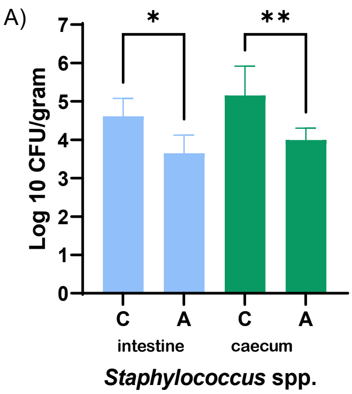

Figure 1. The effect of alfalfa meal on the colony forming units (CFU) of *Staphylococcus* spp. in broiler chickens intestine and caecum

The *Enterococcus* spp. (Figure 2) was significantly altered only in the intestine segment while the *Coliforms* (Figure 3) were significantly decreased only in the caecum segment.

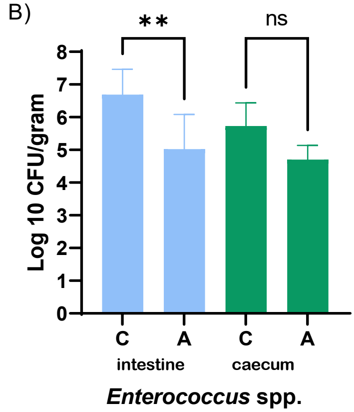

Figure 2. The effect of alfalfa meal on the colony forming units (CFU) of *Enterococcus* spp. in broiler chickens intestine and caecum

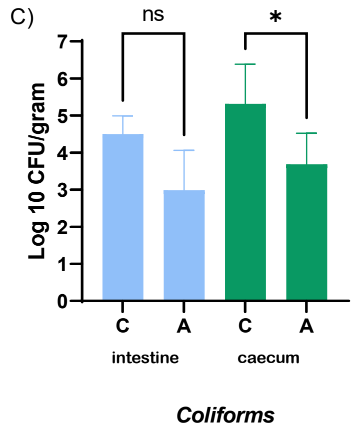

Figure 3. The effect of alfalfa meal on the colony forming units (CFU) of *Coliforms*, in broiler chickens intestine and caecum

No effect was observed for *Clostridium* spp. (Figure 4) while the CFU of *Escherichia coli* (Figure 5) were absent in both segments of the group A compared with the group C, where only the caecum was absent.
The total count of *Enterobacteriaceae* (Figure 6) was lower in both intestinal segments of the group A, but a significant effect was noted only in the caecum.

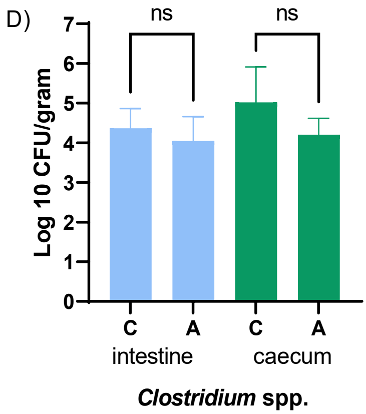

Figure 4. The effect of alfalfa meal on the colony forming units (CFU) of *Clostridium* spp. in broiler chickens intestine and caecum

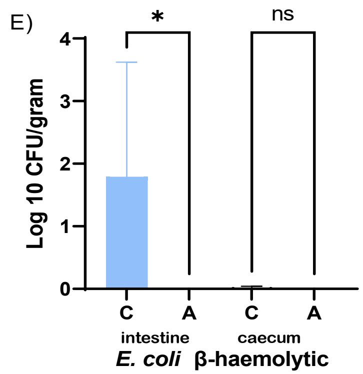

Figure 5. The effect of alfalfa meal on the colony forming units (CFU) of *Escherichia coli* in broiler chickens intestine and caecum

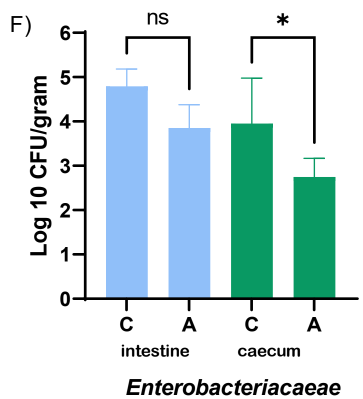

Figure 6. The effect of alfalfa meal on the colony forming units (CFU) of *Enterobacteriaceae* in broiler chickens intestine and caecum

Due to the numerous bioactive compounds present in the alfalfa meal, the CFU of beneficial bacteria determined in this study (*Lactobacillus* spp.) was significantly increased in the intestine and caecum (Figure 7). Although the alteration of the microflora population was made by the bioactive compounds present in the added feed ingredient, however, the composition of the intestinal microbiota can be also altered by good environmental conditions.

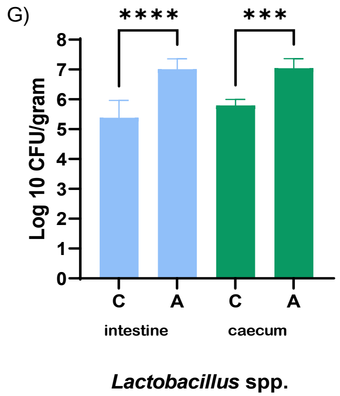

Figure 7. The effect of alfalfa meal on the colony forming units (CFU) of *Lactobacillus* spp. in broiler chickens intestine and caecum

It has been reported that plant bioactive compounds can be fermented to produce metabolites by the gut microbiota in the distal gastro intestine, and further change and reshape the intestinal microbial community via fermentation (Zhang et al., 2022). In line with our findings, it was reported by Varzaru et al. (2020), that the addition of 5% alfalfa meal had a significant effect on altering the microbial population in the intestine and caecum. Other authors (Cui et al., 2022) reported that dietary inclusion of 6% alfalfa was beneficial to improve the small intestinal morphology, caecal microbiota diversity and caecal metabolic function in Zhuanghe Dagu chickens These results were previously sustained by Zheng et al. (2019), when alfalfa meal was tested during the growing phase of the broiler chickens. The beneficial effects have been also supported in other studies when different plant feed additives have been used in broiler diets to promote the health of intestinal microbiota (Turcu et al., 2018; Saracila et al., 2018;

Panaite et al., 2022). In one of the above-mentioned papers, it was reported that obtaining different results among different parts of the microbiota (intestine or caecum) is normal because each part of the intestine develops its unique microbial profile. Inulin and citrus wastes supplementation were also reported to be efficient in decreasing the intestinal and caecal CFU of *Escherichia coli*, and *Salmonella*, promoted the proliferation of *Lactobacillus* spp. in broilers (Yusrizal et al., 2003; Vlaicu et al., 2020). To date the main explanation is given by the mechanism of pathogen inhibition in the microbiome which includes the competition for nutrients and binding locations on the intestinal epithelium, creating short-chain fatty acids and decreasing the pH (Yaqoob et al., 2021). Lower intestinal pH, improve the intestinal microbial balance by reducing the population of pathogenic species, and thus improves the health of the host, resulting in improved performances and health, without affecting these parameters. Although probiotics still have a top place as feed additives to replace antibiotics usage (Yaqoob et al., 2021; Lefter et al., 2023), the need for new dietary ingredients makes the plants a significant competitor to them.

CONCLUSIONS

Alfalfa meal can be used safely for up to 5% in broiler chickens' diets, during the grower and finisher phases, without altering the body weight and with a beneficial effect on some edible carcass parts and intestinal microbiota. However, alfalfa meal can significantly affect the feed conversion ratio, due to anti-nutritional factors.

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VITAMIN AND MINERAL NUTRITION OF DAIRY COWS AND ITS INFLUENCE ON RUMINAL METABOLISM AND MILK PRODUCTIVITY

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Abstract

The key to the intensive course of metabolic processes in the body of ruminants, and therefore to their high productivity, is the balance of rations according to the optimal level of vitamin and mineral nutrition, which is achieved through the use of balancing feed supplements. Providing the animal body with the necessary nutrients and biologically active substances determines not only the level of productivity, but also the amount of feed costs per unit of production, which ultimately determines the profitability of the industry. Therefore, the basis of the planned research was to find out the influence of different levels of vitamin and mineral nutrition on the ruminal metabolism of cows and their milk productivity. Based on the results obtained in the course of the research, the prospective use of the improved vitamin-mineral supplement in the composition of compound feed K 60-32-89 (optimized for Phosphorus and Sulfur) was experimentally confirmed in the feeding of dairy cows during the summer grazing period. A balancing feed supplement is enriched with biologically active substances that are deficient for Pre-Carpathia in a complex with improved compound feed it provides the optimal level of vitamin and mineral nutrition of ruminants in accordance with the physiological need, which contributes to increasing the nutritional value of feed and has a positive effect on the studied indicators of ruminal metabolism. In particular, feeding dairy cows with optimized vitamin-mineral supplement contributes to an increase in the number of microorganisms (amylo-, cellulose- and proteolytic) in the forestomachs and their enzymatic activity. This causes intensive hydrolysis of feed carbohydrates and increases the level of volatile fatty acids by 14.1% while reducing ammonia nitrogen by 11.8%, which is evidence of the activation of metabolic processes involved in energy and synthetic reactions. By analogy with the increase in the intensity of metabolic processes in the rumen of ruminants as a result of a balanced diet by the limiting biologically active substances due to the use of vitamin-mineral supplement, the level of milk productivity increases by 10.8% and the chemical composition of milk improves (dry matter, fat, protein, milk sugar, calcium) compared to the P 60-5M premix.

***Key words**: dairy cows, milk productivity, premix, rumen content, vitamin-mineral supplement.*

INTRODUCTION

Animal husbandry is a strategically important branch of agriculture that saturates the market with food products, industrial enterprises – with the raw material base for production, that is, it acts as a guarantee of food independence and security of the country (Bryk, 2018). The most effective industry, which provides the population with more than 2/3 of animal protein, is dairy farming (Bozhydarnik, 2010).

The profitability of dairy farming is determined by the body's ability to effectively transform feed nutrients into products, which is closely related to the intensive course of metabolic processes in the body at all levels of vital activity (Yanovych & Solohub, 2000; McDonald et al., 2011; Antypin et al., 2014). The key to achieving maximum conversion of feed substances into products is a complete and balanced livestock nutrition system according to scientifically based norms, which would create the most optimal conditions for stimulation of anabolism processes (Spears, 2011; Kozyr et al., 2014; Diachenko et al., 2015). An important role among nutritional factors is the issue of providing rations with the optimal amount and ratio of biologically active substances, in particular, mineral elements and vitamins, etc. The latter affect energy, protein, carbohydrate and lipid metabolism, act as catalysts and cofactors of biochemical processes, contribute to the degradation, assimilation and reduction of consumption of feed nutrients associated with the process of their conversion into products (Nocek et al., 2006; Suttle, 2010; Vorobel & Pivtorak, 2011). The analysis of literary sources shows that the deficiency, excess and imbalance of mineral elements and vitamins in the rations of ruminants negatively affects the functioning of the entire physiological and biochemical system which ensures the vital activity of the organism as a whole (Antypin et al., 2014; Prylipko & Koval, 2021). Numerous scientific studies indicate a deficiency in the soils of most farms in the Western region of a number of macro (phosphorus, sulfur) and microelements (copper, zinc, cobalt, iodine, selenium) and fat-soluble vitamins (A, D), which, accordingly, affects their content in feed, and therefore also in ruminant rations (Kravtsiv et al., 2001; Rusyn, 2009). The solution to this problem is due to the inclusion in the structure of livestock rations of multi-ingredient feed supplements, enriched with these elements and developed taking into account zonal features and the specifics of the forage base structure (Voitovych & Vovk, 2009; Kafliovska & Bihun, 2012; Sosnovska, 2017; Savchuk et al., 2019). Considering the above, the development and application of feed supplements in the feeding of dairy cows, balanced according to the deficient nutrients of a specific biogeochemical province, is of important scientific and practical significance and requires in-depth study and scientific interpretation.

The purpose of the research was to find out the effectiveness of different levels of vitamin and mineral nutrition on ruminal metabolism in the body of dairy cows in the conditions of Pre-Carpathia and their relationship with the level of milk productivity.

MATERIALS AND METHODS

In order to establish the effective action of different levels of vitamin and mineral nutrition a study was conducted at LLC "Litynske" of the Lviv region during the summer grazing period on two groups of Simmental cows, 10 cows in each: I - control group, II - experimental group. Animals in groups were selected by the method of analogues, taking into account origin, age, live weight, lactation and productivity. The ration of cows - grass-concentrate type. Animal feeding was provided in accordance with scientifically based norms (Bohdanov et al., 2012). Fodder was fed to the animals twice a day: in the morning and in the evening (50% of the total nutrition of the ration). Cows were milked twice, by machine.

The experimental part of the research lasted 120 days, of which the equalization period was 30 days and the main one was 90 days. During the equalization period the animals of both groups received the standard combined feed K 60-32-89 in a complex with the premix P 60-5M along with the feed of the main ration - pasture grass, green mass of cereal-legume mixtures of the green conveyor, cereal-various grass hay and molasses. Combined feed K 60-32-89 was represented by the following ingredients - cereal grain (wheat, barley, oats) and wheat bran. The macroelement composition of the combined feed includes monocalcium phosphate, magnesium oxide and common salt. Premix P 60-5M contains fat-soluble vitamins (A, D), trace elements (Zinc, Cobalt, Iodine) and wheat bran as a filler.

In the main period, dairy cows of the control group were on the ration of the equalization period. The animals of the experimental group received similar feeds with the only difference that the combined feed K 60-32-89 (improved in terms of phosphorus and sulfur) included vitamin-mineral supplement in a similar amount (1%) (to replace the premix P 60-5M). The constituent components of the experimental combined feed are identical to the control, with the additional inclusion of Glauber's salt and a larger amount of monocalcium phosphate. The vitamin-mineral supplement contains copper and selenium and is adjusted for the content of microelements limited in the Pre-Carpathian zone - zinc, cobalt, iodine and fat-soluble vitamins: A, D and the filler is wheat bran.

The material for research was feed, rumen content and milk. In the fodder of the ration, level of macro-, microelements and vitamins were studied. In the ruminal content the following were determined: the content of raw biomass and absolutely dry matter of bacteria, the number of amylo-, cellulose- and proteolytic microorganisms and their activity, the level of volatile fatty acids, pH, the concentration of nitrogen fractions (total,

residual, protein and ammonia) and phosphorus (total acid-soluble, inorganic and organic, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA)). The investigated indicators in the selected samples were determined according to generally accepted methods (Vlizlo et al., 2012).
Statistical analysis of the research results was carried out using the methods of variational statistics in the standard Microsoft Excel and AtteStat application program package using the Student's t-test. Arithmetic mean values (M) and arithmetic mean errors (m) were calculated. Differences between mean arithmetic values were considered statistically significant for: *P<0.05; **P<0.01; ***P<0.001.

RESULTS AND DISCUSSIONS

Based on the obtained results it was established that during the main period no significant deviations between the groups in the amount of physical feed consumed by dairy cows were found. There is also no difference in a number of nutrients - metabolic energy, dry matter, crude and digestible protein, crude fat, crude fiber, sugar at the optimal ratio of sugar to protein (1.0: 1.0), carbohydrates to protein (2.0: 1.0), calcium to phosphorus (1.9-2.0: 1.0), nitrogen to sulfur (11.2-12.0: 1.0), however, there is a different biologically active substances supply in rations. Therefore, it should be noted that the main factor of the productive effect, i.e. the level of metabolic processes in the animal body and their connection with productivity, in the list of ration components were compound feed K 60-32-89 (standard and improved), premix P 60-5M and vitamin-mineral supplement.
Analysis of the chemical composition of feed in the rations of cows of the control group indicates a lack of phosphorus, sulfur, copper, zinc, cobalt, iodine, selenium, vitamins A and D, i.e. combined feed K 60-32-89 in a complex with premix P 60-5M does not provide optimal level of vitamin and mineral nutrition. At the same time, in the rations of animals of the research group, their deficiency was replenished by feeding vitamin-mineral supplement, adjusted for the content of limiting microelements and fat-soluble vitamins in the composition of compound feed, improved for phosphorus and sulfur. each of the above feeding factors, individually and in combination, plays a certain role in the chain of exchange processes in the livestock organism, and this, accordingly, affects the level of its productivity.
The efficiency of feed utilization by ruminants depends significantly on the nature of ruminal fermentation. It is known that organic feed compounds that enter the gastrointestinal tract of livestock are in a complex form and are broken down into simple compounds (which, in turn, are assimilated by microorganisms) are disposed of in the forestomachs (Antypin et al., 2014).
The key factor determining the intensity of ruminal metabolism in dairy cows is the pH value. This indicator in the animals of the experimental group (Table 1) is slightly lower compared to the control (by 1.7%), i.e. shifted to the acidic side.
The analysis of the level of metabolic processes in the rumen of ruminants indicates an increase in the concentration of microorganisms during feeding of vitamin-mineral supplemen. In particular, the number of amylolytic bacteria in the rumen content of the experimental group exceeds the control group by 16.7% (P<0.05). In the rumen environment of animals of the II group, an increase in the number of cellulolytic microflora is observed - by 20.5% and, according to statistical data, it is probable (P<0.05). The concentration of proteolytic microorganisms in the cows of the experimental group increases by 14.7%, compared to the control.
An increase in the number of ruminal biota in ruminants on the background of the vitamin-mineral supplemen use indicates the intensification of the processes of division and reproduction of bacterial cells, their growth, and therefore, the active accumulation of raw biomass in the forestomach. This ruminal metabolite increases in the experimental group of animals compared to the control by 13.3%, and the probability criterion is P<0.05. The largest share in the biomass is represented by the amylolytic population, the number of which in the rumen is the highest, relative to cellulolytic, proteolytic, etc. At the same time, in the raw bacterial mass of the II group, the content of absolutely dry matter increases by 1.4 times compared to the control.

Table 1. Indicators of ruminal metabolism of dairy cows (M ± m, n = 3)

The investigated indicator and unit of measurement	Groups of animals	
	I (control)	II (experimental)
pH	6.93±0.04	6.81±0.03
Raw biomass of bacteria, mg/100 ml	1057.0±33.8	1197.0±12.0*
Dry biomass of microflora, mg/100 ml	175.0±12.2	243.0±11.1*
Number of microorganisms, million/ml:		
amylolytic	10.20±0.22	11.90±0.37*
cellulolytic	6.00±0.33	7.23±0.21*
proteolytic	3.60±0.12	4.13±0.12*
Enzyme activity:	9.73±0.29	11.10±0.25*
amylolytic, conditional amylolite unit	1.27±0.04	1.65±0.05**
cellulolytic, %	15.94±0.90	21.10±0.92*
proteolytic, Mekv. tyrosine in 100 ml/min	0.281±0.012	0.293±0.01
VFA, mmol/100 ml	9.73±0.29	11.10±0.25*
Nitrogen, mmol/l		
total	80.79±0.44	86.29±0.44***
residual	22.73±0.14	24.15±0.44*
protein	58.06±0.55	62.14±0.83*
ammonia	13.36±0.20	11.79±0.21**
Phosphorus, mmol/l		
total acid-soluble	10.02±0.16	10.99±0.18*
inorganic	7.39±0.07	7.06±0.05*
organic	2.63±0.13	3.93±0.23**
RNA	617.2±20.0	701.9±6.7*
DNA	396.1±21.5	438.3±6.3

Note. The difference is probable regarding control: *P<0.05; **P<0.01; ***P<0.001.

Thus, the listed ruminal metabolites are a confirmation of the synthesis of an easily digestible, biologically valuable microbial protein, which is nutritionally equivalent to chicken egg protein. At the same time, the microbial protein is enriched with sulfur-containing amino acids (methionine, cystine, cysteine) in the rations of the research group's ruminants at an optimal level of sulfur. Microorganisms' protein assimilation is on average 70-80%, digestibility - 80-86%.

In the content of the rumen of cows of the II group after feeding vitamin-mineral supplemen, compared to the premix P 60-5M, simultaneously with the change in the number of the listed species of bacterial populations in the forestomachs, a similar pattern occurs with their enzymatic activity. In the rumen fluid of animals of the experimental group, relative to the similar indicator of the control group, amylase activity is higher by 29.9%, and it is probable (P<0.01). In this experiment, the activity of cellulolytic microflora also increases in the rumen of cows of the II group, in particular, by 5.2%, and according to statistical data, the difference is probable (P<0.05). The advantage of the enzymatic activity of proteolytic bacteria in the rumen fluid of animals of the research group is 4.3%. The increase in the activity of microorganisms in the contents of the rumen of cows of the II group contributes to the intensive splitting of structural (cellulose, hemicellulose, pectin, etc.) and non-structural (starch, sugar) carbohydrates of feed, which causes the formation of a large amount of the final product - volatile fatty acids (acetic, propionic, butyric and a small amount of formic, isobutyric, valeric and caproic acid) - by 14.1% (P<0.05). Volatile fatty acids, on the one hand, serve as the main source of metabolic energy (glycolysis) and after absorption in the rumen are used in the body for energetic (Krebs cycle) and synthetic processes, as well as precursors of milk components - fat and protein, and on the other hand - valeric, caproic, isobutyric and isovaleric acids stimulate the breakdown of fiber and assimilation of ammonia by bacteria. At the same time, the listed acids are carriers of the carbon skeleton involved in protein synthesis by the microbial cell (Obertiukh, 2005).

Nitrogen and Phosphorus fractions are actively included in the process of intensive ruminal metabolism in the body of animals of the experimental group. In particular, in the content of the rumen of cows of the II group, there is an increase in the concentration of total nitrogen, relative to I group by 6.8% ($P<0.001$). Residual Nitrogen in the rumen fluid of animals of the experimental group exceeds the similar indicator of the control group by 6.2% and is within the limits of probability ($P<0.05$). On the background of feeding improved vitamin-mineral supplement in the rumen content of cows, compared to premix P 60-5M, the concentration of protein nitrogen increases by 7.0% ($P<0.05$). Ammonia is an important indicator of fermentation in ruminant forestomachs, which for most microorganisms (about 90%) is one of the main sources of Nitrogen in the processes of microbial protein synthesis, and for 25% it is an indispensable factor for bacterial growth. The concentration of this fraction in the rumen content of cows of group II is lower by 11.8%, and according to statistics, the difference is probable ($P<0.01$). The indicated level of ammonia nitrogen in the rumen fluid of animals is a consequence of its effective use by the microorganisms of the forestomachs for the synthesis of the main components of their body. The most active "consumers" of ammonia are amylo- and cellulolytic microflora, which is consistent with their high concentration in the rumen environment of cows in the study. It is known that an acidic environment helps reduce the intensity of ammonia absorption by the rumen wall, i.e. prolongs the period of the latter's stay in the forestomach, which thereby enables bacteria to absorb it as efficiently as possible (Antypin et al., 2014).

Phosphorus is an indispensable element in the processes of glycolysis and the Krebs cycle, due to which macroergic compounds (ADP, ATP, ATP + ADP, etc.) are formed. These compounds are universal accumulators and energy donors, which ensures the normal functioning of the bacteria of the forestomach and the macroorganism as a whole. The structure of nucleic acids, which are carriers of genetic information, includes orthophosphate, which is involved in the regulation of protein biosynthesis. The level of rumen phosphorus, in turn, is closely related to vitamin D. The latter increases the activity of microbial phytases, which enhance the hydrolysis of inositol phosphates with the release of inorganic phosphorus (Demydiuk et al., 2011). In this experiment, the amount of total acid-soluble Phosphorus in the rumen content of animals of the II group exceeds this indicator of I group by 9.7% ($P<0.05$). The level of inorganic Phosphorus in the rumen fluid of cows of the experimental variant is probably lower (by 4.5%; $P<0.05$), due to its active use by bacteria in the processes of glycolysis and anabolism. On the background of improved vitamin-mineral supplement in the rumen content of ruminants there is a probable increase in organic phosphorus by 1.5 times. In the rumen fluid of cows of the II group the content of phosphorus RNA is higher - by 13.7% ($P<0.05$), compared to I group. The concentration of phosphorus DNA in the rumen content of the animals of the experimental group exceeds the similar indicator of the control group by 10.7%.

The data obtained in the experiment are to some extent agree by the results of a similar research (Snitynskyi et al., 2009; Kafliovska and Bihun, 2012; Sosnovska, 2017; Sachuk et al., 2019). Thus, the use of the complex mineral preparation "Kalfomin" prevents the occurrence of cow diseases associated with metabolic disorders, in particular postpartum paresis, and in young animals - rickets (Sachuk et al., 2019). Research by Sosnovska (2017) found that feeding premix to dairy cows contributes to a more complete assimilation of nutrients in the ration and thus leads to an increase in milk yield, depending on its amount - 1% and 1.5%, respectively by 0.2 and 0.4 kg. Other scientists also found a positive effect of premixes on the viability of calves and their higher resistance to dyspeptic diseases, and in the blood serum of cows during the dry period, there was an increase in the content of vitamin A (Kafliovska and Bihun, 2012).

The analysis of the conducted studies shows that feeding dairy cows with improved vitamin-mineral supplement in a complex with improved compound feed provides an optimal level of vitamin and mineral nutrition, which contributes to an increase in the intensity of the metabolic processes in the rumen, and as a result of this - an increase of the milk productivity by 10.8%. At the same time - improvement of the chemical composition of milk, in particular, an increase in fat and protein, respectively by 0.1% and 0.12%.

CONCLUSIONS

It is theoretically substantiated and experimentally proven, taking into account the level of metabolism in the body of cattle and their milk productivity, that for the purpose of profitable dairy farming in the soil and climate zone of Pre-Carpathia, it is recommended to feed dairy cows with improved by phosphorus and sulfur compound feed. the use of vitamin-mineral supplement in livestock feeding contributes to the increase in the nutritional value of feed, the activation of metabolic processes in the body and as a final result obtaining of high-quality products at a low cost.

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MORPHOFUNCTIONAL FEATURES OF MECKEL'S DIVERTICULUM OF GEESE

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Abstract

The parameters and dynamics of the development of Meckel's diverticulum in large gray geese aged one day to 5 years were determined. The changes in the length, cross-sectional area and wall area of the Meckel's diverticulum indicate that its growth stops by 3 months of age. The age-related morphofunctional indicators of lymphoid tissue formation in the wall of Meckel's diverticulum were determined. Diffuse lymphoid tissue is predominant in the composition of lymphoid tissue. Full morphofunctional maturity of the lymphoid tissue with the development of four levels of its structural organization is observed at the age of 21 days in geese. The lymphoid tissue in the wall of Meckel's diverticulum reaches its maximum development by the age of 3 months, which must be taken into account when raising large gray geese and conducting experimental studies.

***Key words**: geese, lymphocytes, lymphoid tissue, Meckel's diverticulum.*

INTRODUCTION

The lymphoid tissue of the mucous membranes makes up more than half of the body's lymphoid tissue, forming their unified immune system (Schuh, 2020; Junior et al., 2018; Kang et al., 2014; Cesta, 2006; Ciriaco et al., 2003; Jeurissen et al., 1994; Kendall, 1980). The lymphoid tissue of the mucous membrane of the digestive tube contains the largest pool of immunocompetent cells: up to 80% of B-lymphocytes, macrophages, antigen-stimulated T-helpers, plasma cells. The formation of immunoglobulin molecules in mucous membranes occurs on the surface of epitheliocytes, where they provide local antibacterial and antiviral protection (Meek et al., 2022; Rehfeld et al., 2017; Day & Schultz, 2014; Rehfeld et al., 2013; Korver, 2006; Friedman et al., 2003). In lymphoid formations of the digestive tube of birds, lymphoid tissue can be located diffusely, forming clusters in the form of lymphoid nodules, tonsils, Peyer's patches, Meckel's diverticulum - lymphoid diverticulum of the jejunum (Khomich et al., 2020; Makhotina et al., 2020; Kushch et al., 2019; Dishluk & Orlova, 2017; Al-Juboury et al., 2016; Kaspers & Göbel, 2016; Kharchenko & Lykova, 2013; Brandtzaeg et al., 2008; Besoluk et al., 2002; Jeurissen et al., 1989).

In 1809, Johann-Friedrich Meckel Jr. (Meckel J.F., 1781-1833) first described the embryonic yolk duct in humans, named Meckel's diverticulum in his honor (Meckel diverticulum) (Ibrahim & Mohamed, 2023; Kafshgari et al., 2023; Farrell & Zimmerman, 2020; Lindeman & Søreide, 2020; Hamilton & Arnason, 2015; Farah et al., 2015; Opitz et al., 2006).

The first reports on the origin, structure and functional significance of Meckel's diverticulum, published in 1984, laid the foundation for its morphological studies in birds (Olah & Glick, 1984; Olah et al., 1984). Features of the morphogenesis of Meckel's diverticulum in the postembryonic period of ontogeny have been studied only in chickens and ducks (Mazurkevych & Khomych, 2017; Khomych & Mazurkevych, 2015; Mazurkevych, 2013a; Mazurkevych, 2013b; Mazurkevych, 2012; Jamroz et al., 2004; Kalynovska, 2004; Bar-Shira et al., 2003; Friedman et al., 2003; Besoluk et al., 2002; Jeurissen et al., 1994; Jeurissen et al., 1989; Olah et al., 1984; Olah & Glick, 1984). In this regard, the features of the development of Meckel's diverticulum in geese, its lymphoid tissue and the levels of its structural organization require in-depth study.

MATERIALS AND METHODS

The material for the study was Meckel's diverticulum from clinically healthy large gray geese 1-, 3-, 7-, 14-, 21-day-old, 1-, 2-, 3-, 6-, 8-month-old and 1-, 2- , 3- and 5-year-old (n = 5). The material was fixed in 8% aqueous neutral formalin and embedded in paraffin. Serial sections 7-8 μm thick were stained with H&E, aniline-blau-orange according to Mallory, Kelemen, Brachet. The total population of endocrinocytes was detected by the Grimelius method. Enterochromaffin EC cells were identified by the Masson-Gamperl method modified by I. Singh. Lymphoid cells were determined on Pappenheim-stained imprint preparations (Horalskyi et al., 2019).

The cross-sectional area of Meckel's diverticulum, its walls, membranes, crypts, and lymphoid formations was defined using a *Jenamed-2* light microscope. *Image Tools 3.6* software was used to determine the morphometric parameters of the microstructure of Meckel's diverticulum. Endocrinocytes were counted using an eyepiece grid, in terms of 1 mm^2 of the mucous membrane. Obtained digital indicators were processed using one-way ANOVA and Student's t-test. The data obtained in the tables are presented as a standard deviation. The reliability of the difference in indicators was determined by the reliability criterion (td) and Student's tables.

The experiment was conducted in accordance with generally accepted principles of humane treatment of animals (Law of Ukraine "On the Protection of Animals from Cruelty Treatment", No. 3447-IV as of 21.02.2006, Kyiv; European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, Strasbourg, 1986). The work was done at the Department of Normal and Pathological Morphology of the State Biotechnological University (Kharkiv, Ukraine).

RESULTS AND DISCUSSIONS

Resulting from the conducted study, it was established that Meckel's diverticulum (MD) in geese in embryogenesis is a derivative of the yolk stalk. In the postembryonic period of ontogenesis, MD is a permanent peripheral organ of the immune system, has a conical shape, and is tubular in structure. It is located on the antimesenteric surface of the loop of the jejunum, its apex is directed caudo-ventrally, and its base communicates with the lumen of the jejunum (Figure 1).

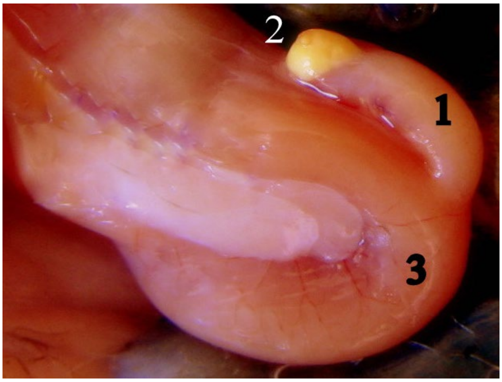

Figure 1. Loop of jejunum with Meckel's diverticulum of a 14-day-old gosling (macropreparation): 1 - Meckel's diverticulum; 2 - the rest of the yolk; 3 - jejunal loop

The length of the segments of the small intestine before and after the MD in geese of all the studied age groups is stable at an average ratio of 0.59: 0.41 cm. The length of the MD from a day- to 2-month-old geese increases in direct proportion to body weight, the total length of the intestine and, especially, the thin section. In the first three months of a bird's life, the length of the MD increases by 3.1 times (17.50 ± 3.50 mm). In 6-month-old geese its length decreases to 13.25 ± 1.55 mm, and from the age of 8 months to 5 years it is in the range of 13.67 ± 0.33 - 12.67 ± 0.33 mm.

The area of the transverse section of the MD by the age of 3 months increases by 34 times (13.84 ± 0.90 mm^2), and the area of the wall of the MD increases by 36 times, reaching a maximum value (12.63 ± 0.82 mm^2). From the age of 3 months to 3 years, the cross-sectional area of the MD decreases by 1.5 times (9.36 ± 0.30 mm^2), and up to 5 years of age - by 1.7 times (7.97 ± 0.12 mm^2). The wall area of the MD of geese up to 3 years of age decreases by 1.4 times (8.92 ± 0.23 mm^2), and up to 5 years of age by 1.8 times (6.84 ± 0.13 mm^2), which indicates a direct correlative dependence in the development of the compared MD structures.

An increase in the area of transverse sections of the MD and its wall up to 3 months of age occurs due to a more intensive development of the mucosa, the formation of cellular structures of the epithelial layer in it, its own plate and submucosa, as well as the formation of folds and crypts that increase the total area of the mucosa, through which the immune system controls the antigens that fall on its surface (Schuh, 2020; Day & Schultz, 2014; Koutsos & Klasing, 2014; Casteleyn et al., 2010; Friedman et al., 2003; Jamroz et al., 2004; Besoluk et al., 2002; Jeurissen et al., 1989). The absolute area of the mucous membrane of the MD by the age of 3 months increases by 65 times (11.63 ± 0.73 mm^2), and the relative area reaches a maximum value of 92.08%. From 6 months to 5 years of age, the absolute area of the mucous membrane decreases to 5.55 ± 0.27 mm^2. The relative area of the mucous membrane of the MD of 5-year-old geese decreases, but remains at a fairly high level of 81.14%.

With age, structural reorganization of the mucous membrane of the MD occurs. Up to 3 days of age, the epithelial layer is represented by a single-layer prismatic border. It defines prismatic cells with a border, goblet, endocrine and cambial cells with mitotic figures. The number of goblet cells in the epithelial layer prevails in 21-day-old geese and persists up to 2 years of age. The secretion of goblet cells of the epithelium act as a protective barrier, it constantly renews the coating of the mucous membranes, promotes the transport of secretory immunoglobulin to its surface, providing the first line of immune defense (Kushch et al., 2020; Yu et al., 2020; Ross & Pawlina, 2015; Day & Schultz, 2014; Besoluk et al., 2002; Sharma, 1991).

In 21-day-old geese, in the total population of endocrinocytes of the epithelial layer, the number of EC cells has a maximum value of 97.66%. From one month to 2 years of age, it is 84.81% and 83.60%, respectively, and in 3-5-year-olds it is 43.40 and 33.73%. Based on the fact that the biologically active substances of EC cells are involved in the regulation of the processes of proliferation, growth, and differentiation of cells in tissues, these processes are reduced in the MD of geese of older age groups (Kushch et al., 2019; Koutsos & Klasing, 2014; Bar-Shira et al., 2003).

In geese up to 3 months of age, branched folds predominate in the MD mucosa, which is associated with the process of crypt formation. Thus, in 21-day-old geese, compared with 1-day-old geese, the area of crypts increases by 11 times, and in 2-month-old geese - by 17.9 times. From the age of 6 months, the folds of the mucous membrane acquire a columnar and triangular shape. The area of crypts in 6-month-old geese, compared to 3-month-old ones, decreases by 1.7 times. At the same time, individual crypts are expanded, their wall is lined with squamous epithelium, they are filled with a thickened oxyphilic secret, which contains lymphocytes, eosinophils and cellular detritus. In geese from 1 to 5 years of age, the crypts are solitary, their lumen is narrowed, and some take the form of cysts. All this leads to a decrease in the source of regeneration of the integumentary epithelium and the total area of the mucous membrane (Doneley, 2016; Samour, 2015; Bar-Shira et al., 2003; Besoluk et al., 2002).

The epithelium of the folds and crypts of the mucous membrane of the MD of geese in the postnatal period of ontogenesis is unevenly infiltrated with lymphocytes. They interact with antigens, performing the function of the first protective barrier, which is typical for lymphoid formations associated with mucous membranes (Junior et al., 2018; Davison, 2014; Ruddle & Akirav, 2009; Bar-Shira et al., 2003; Friedman et al., 2003).

In the lamina propria of the mucous membrane of the MD of 1-day-old geese, diffuse lymphoid tissue (LT) is formed, structures of loose fibrous connective tissue and vessels of the microvasculature are formed. Reticulocytes, lymphocytes, macrophages, eosinophils, reticular fibers and single fibroblast cells are detected in the lamina propria. Lymphocytes are predominant in the cell population. Single lymphocytes are found intraepithelially and on the surface of the mucous membrane. The absolute area of the LT is 0.176 ± 0.009 mm^2, and the relative area is 50.30%. (Figures 2, 3).

With the age of geese, diffuse LT gradually occupies the entire area of the mucosal lamina propria. In 3-day-old geese, compared to 1-day-old geese, the LT occupies 58.00% of the wall area of the MD. Its absolute area increases by 4.3 times (0.76 ± 0.02 mm^2).

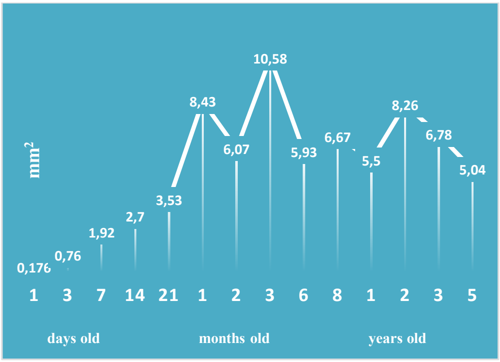

Figure 2. The absolute area of lymphoid tissue in the wall area of Meckel's diverticulum of geese, mm^2

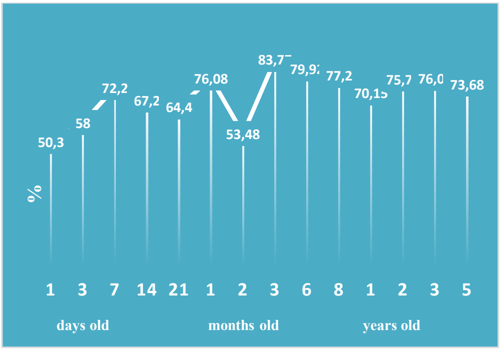

Figure 3. The relative area of lymphoid tissue in the wall area of Meckel's diverticulum of geese, %

As part of diffuse LT denser accumulations of lymphocytes in the form of prenodules (3-4 per section area) are detected for the first time. Around the crypts, single primary lymphoid nodules are formed, which are an integral part of the immune structures associated with the mucous membrane (Nochi et al., 2018; Kaspers & Göbel, 2016; Day & Schultz, 2014; Randall et al., 2008; Friedman et al., 2003). They are based on reticular tissue populated by small lymphocytes, eosinophils and single macrophages. The periphery of primary lymphoid nodules is surrounded by fibroblasts and single collagen fibers.

From the age of 7 days, LT is detected in the mucous, muscular and serous membranes of the MD. Diffuse LT, prenodules and primary lymphoid nodules continue to be formed in the mucosa. The absolute area of the lymphoid tissue increases by 11 times (1.92 ± 0.04 mm^2), and the relative area by 14.20%, up to 72.20%. Lymphocytes are detected both intraepithelially and on the surface of the mucous membrane of the MD. Diffuse LT fills the subepithelial areas of the main mucosal lamina. In the deep layer of the lamina propria around the crypts, prenodules and primary lymphoid nodules are formed, surrounded by a connective tissue membrane. In the muscular and serous membranes, diffuse LT is detected around the blood vessels.

In 14-day-old geese, LT is represented by a diffuse form, prenodules, and emerging primary lymphoid nodules. The relative area of LT decreases to 67.20%, which is due to an increase in the area of the crypts (17.93%). The absolute area of LT increases by 1.4 times (2.70 ± 0.12 mm^2). Lymphocytes are detected intraepithelially, on the surface of the mucous membrane, as well as in the secretion of crypts. Primary lymphoid nodules are found in the deep layer of the lamina propria of the MD mucosa. In the muscle membrane, diffuse LT is detected between the muscle layers. Primary lymphoid nodules appear in the serous membrane of the MD.

In 21-day-old geese, LT in the MD wall is represented by a diffuse form with prenodules, primary and secondary lymphoid nodules, which is an indicator of the full morphofunctional maturity of the MD as a peripheral organ of immunogenesis. The absolute area of LT increases (3.53 ± 0.21 mm^2), while the relative area decreases (64.40%), which is associated with an increase in the area of the crypts. Lymphocytes are detected on the surface of the mucous membrane, interepithelially and as part of the secretion of crypts. In the surface layer of the main plate of the mucous membrane, lymphocytes are located diffusely, in some places very densely. The total area of diffuse LT (87.00%) significantly exceeds the area of lymphoid nodules (13.00%). Formed primary and solitary secondary lymphoid nodules are revealed in the deep layer of the lamina propria (Figure 4).

In the central, light part of the secondary lymphoid nodules, the cells are concentrated in groups, in the form of "rosettes". In the center of each of them is a macrophage surrounded by lymphocytes, which indicates antigen presentation (Figure 5).

Between the rosettes, a reticular skeleton is clearly visible, in which small, medium and large lymphocytes with pyroninophilic cytoplasm are located. The periphery of secondary lymphoid nodules is populated with small lymphocytes in 4-5 rows. In the muscle membrane, diffuse LT is detected between the layers of muscle bundles. In places, lymphocytes surround the blood vessels, forming clusters in the form of "couplings". Single primary lymphoid nodules are formed on the mesenteric surface of the serous membrane of the MD, around the blood vessels.

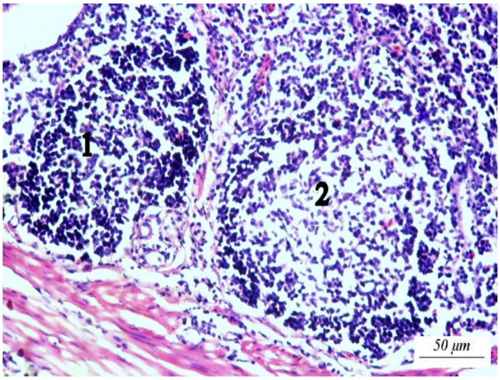

Figure 4. Primary (1) and secondary (2) lymphoid nodules in the lamina propria of the mucosa of Meckel's diverticulum of a 21-day-old gosling (H & E)

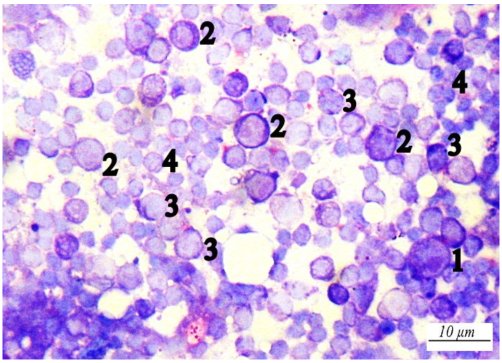

Figure 5. The central part of the secondary lymphoid nodule: 1 - a macrophage in the center of the "rosette"; 2 - large lymphocytes; 3 - medium lymphocytes; 4 - small lymphocytes. Pappenheim staining

In one-month-old geese, the absolute (8.43 ± 0.48 mm^2) and relative (76.08%) area of LT in the MD wall increases. Diffuse LT predominates in the proper layer of the mucous membrane and around the crypts (82.90%) with denser accumulations in the form of prenodules. Lymphoid nodules (17.10%) are detected in the deep layer of the lamina propria of the mucous membrane, the submucosa, between the layers in the muscular and serous membranes. In the center of individual lymphoid nodules, cells with mitotic figures, as well as plasma blasts and plasma cells, are detected. There is antigen presentation, proliferation and differentiation of cells characteristic of the antigenic response and the formation of secondary lymphoid nodules.

In two-month-old geese, compared to one-month-old geese, the absolute (6.07 ± 0.31 mm^2) and relative area of LT (53.48%) decreases, which is associated with an increase in the area of crypts (39.66%). Diffuse LT and lymphoid nodules account for 85.60% and 14.40% of the total LT area, respectively. Diffuse LT is formed subepithelially in its own layer of the mucous membrane and around the crypts. Primary lymphoid nodules are located in the deep layer of the lamina propria, and secondary, in addition to the deep layer of the lamina propria, are concentrated in the submucosal layer of the mucosa, between the layers in the muscular and serous membranes.

In three-month-old geese, the absolute (10.58 ± 0.65 mm^2) and relative area (83.77%) of LT increase. At the same time, the area of diffuse LT decreases (54.90%), while the area of lymphoid nodules increases (45.10%). An increase in these morphological components is considered an indicator of the activation of the main function of the organ (Kaspers & Göbel, 2016; Fellah et al., 2014; Ruddle & Akirav, 2009; Bar-Shira et al., 2003). Diffuse lymphoid tissue with prenodules is concentrated in the lamina propria. Primary lymphoid nodules (17.00%) are located in the deep layer of the lamina propria, in the submucosa of the mucous membrane, as well as in the muscular and serous membranes (Figure 6).

Secondary lymphoid nodules (28.10%) are concentrated mainly in the deep layer of the lamina propria and the submucosa of the mucosa. In 3-month-old geese, the structures of immune protection in the wall of the MD reach full and maximum development, which must be taken into account when growing large gray geese.

From the age of 6 months, both quantitative and qualitative indicators of LT noticeably change. Its absolute (5.93 ± 0.29 mm^2) and relative (79.92%) area decreases. The relative area of diffuse LT increases (85.50%). It is concentrated in its own layer of the mucous membrane subepithelially and around the crypts. The relative area of lymphoid nodules is 14.50%. Primary lymphoid nodules predominate in number, located in the deep layer of the lamina propria, the submucosa of the mucosa, and between the layers in the muscle membrane. Secondary lymphoid nodules are longitudinally oval in shape, concentrated in the deep layer of the lamina propria and the submucosa of the mucosa.

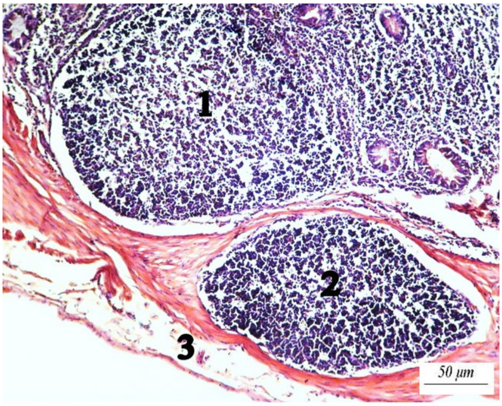

Figure 6. A primary lymphoid nodule (1) in the submucosal base of the mucosa and in the muscle sheath (2) of the wall of Meckel's diverticulum of a 3-month-old gosling; 3 - serous membrane (H & E)

In accordance with the biological characteristics of the large gray geese, 8 months of age is considered to be the period of reaching puberty. The absolute area of LT, compared to 6-month-old ones, slightly increases (6.67 ± 0.41 mm^2), while the relative area decreases (77.20%). The relative area of diffuse LT decreases (84.30%), while that of lymphoid nodules increases (15.70%). Formed primary lymphoid nodules are localized in the deep layer of the lamina propria and the submucosa of the mucous membrane, and in the muscular and serous membranes they are placed one after another, creating ribbon-like formations. The number of secondary lymphoid nodules increases, which are localized in the deep layer of the lamina propria and submucosa. Next to some secondary lymphoid nodules, bud-shaped formations from lymphocytes are determined and are the beginning of the formation of primary lymphoid nodules.

In one-year-old geese, the absolute (5.50 ± 0.29 mm^2) and relative (70.15%) area of LT decreases. Diffuse LT predominates (80.70%) in the main plate of the mucous and muscular membranes of the MD. The quantitative ratio of primary (14.20%) and secondary (5.10%) lymphoid nodules changes. Primary lymphoid nodules are spherical in shape, located in the deep layer of the lamina propria and muscle membranes, and secondary ones are detected in the submucosa of the mucous membrane.

In 2-year-old geese, the absolute (8.26 ± 0.52 mm^2) and relative (75.71%) area of LT in the MD wall slightly increased. However, the relative area of diffuse LT decreases (76.00%), while the area of lymphoid nodules increases (24.00%). The relative area of primary lymphoid nodules (20.60%) prevails over secondary ones (3.40%). Primary and secondary nodules are predominantly oval in shape. In the deep layer of the lamina propria, the mucous membranes are placed singly. They have a ribbon-like placement in the submucosal layer of the mucosa, in the muscular and serous membranes (Figure 7).

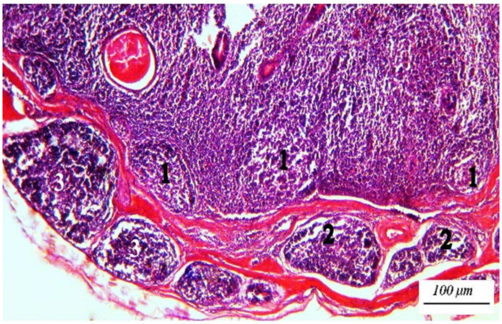

Figure 7. Primary lymphoid nodules in the wall of Meckel's diverticulum of a 2-year-old goose: 1 - in the lamina propria of the mucous membrane; 2 - in the muscular membrane; 3 - in the serous membrane (H & E)

In 3-year-old geese, the absolute LT area in the MD wall slightly decreases (6.78 ± 0.35 mm^2), while the relative area increases (76.01%). Diffuse LT (88.50%) is located in the main lamina of the mucous membrane subepithelially, around the crypts, in the interlayers between the layers of the muscular membrane. Single primary lymphoid nodules (7.08%) of an oval shape are located in the submucosa of the mucosa. Secondary lymphoid nodules (4.42%) are located in the deep layer of the main lamina and the submucosa of the mucosa.

In 5-year-old geese, the absolute (5.04 ± 0.20 mm^2) and relative (73.68%) LT area decreases. In the MD wall, the diffuse form prevails (84.00%) with single prenodules, which in the mucous membrane is located in its own layer, and mainly around the blood vessels in the muscular one. Primary lymphoid nodules (8.90%) are located in the deep layer of the lamina propria, in the submucosal layer of the mucosa, and in groups of 2-3 in the muscular membrane. Secondary lymphoid nodules (7.10%) are longitudinally oval in shape, concentrated in the submucosal base of the mucosa.

CONCLUSIONS

The conducted studies determined the parameters and dynamics of the development of Meckel's diverticulum of the jejunum in large gray geese from 1-day-old to 5 years of age. It has been established that the growth of Meckel's diverticulum stops by the age of 3 months. In the postnatal period of ontogenesis in the Meckel's diverticulum, an age-related structural and functional reorganization occurs, manifested by a change in the structure of crypts and their area, the quantitative composition of cells in the integumentary epithelium, and the form of development of lymphoid tissue. Up to 2 years of age, goblet cells predominate in the integumentary epithelium of the mucous membrane of the Meckel's diverticulum. A decrease in the number of EC cells in the total population of endocrinocytes of the epithelial layer of the mucous membrane in geese of 3 and 5 years of age is associated with a decrease in the processes of proliferation, growth, and differentiation of cells in the tissues of the Meckel's diverticulum wall.

Lymphoid tissue of the mucous membrane of Meckel's diverticulum in its diffuse form is detected from 1-day-old geese. In 3-day-old geese, prenodules appear in diffuse lymphoid tissue, and centers of formation of primary lymphoid nodules appear around the crypts. In 7-day-old geese, lymphoid tissue is represented by a diffuse form with prenodules and emerging primary lymphoid nodules. In 14-day-old geese, the diffuse form, prenodules, and first formed primary lymphoid nodules are detected in the lymphoid tissue of the Meckel's diverticulum. Full morphofunctional maturity of the lymphoid tissue in the Meckel's diverticulum with the development of four levels of its structural organization was recorded in 21-day-old geese. In geese of 3 months of age, the structures of lymphoid tissue reach their maximum development, which must be taken into account when raising geese, forming production groups, carrying out diagnostic, preventive and therapeutic measures, as well as when studying the mechanism of action of immunomodulatory biological products. From 6 months to 5 years of age, the relative area of diffuse lymphoid tissue significantly exceeds the area of lymphoid nodules.

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BREWER YEAST MANNOPROTEINS AS AN EFFICIENT SUPPLEMENT FOR PRESERVATION OF RAM SPERM BY REFRIGERATION

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Abstract

The purpose of the work was to evaluate the influence of a mannoprotein extract (MP) from brewer yeast on the motility, speed, as well as the morphological and microbiological indices of the ram semen preserved by refrigeration at +4°C. The experimental extender (EE) supplemented with MP at the concentrations of 0.6-0.9% v/v significantly increased ($P \leq 0.05$) the total motility (TM) and the progressive motility (PM) of ram spermatozoa during 96 hours of storage and did not negatively influence the VAP, VSL and VCL indices. At the end of the 96-120-hour period of storage MP in the concentrations of 0.8-1.0% v/v contributed to reduction of the abnormal sperm (AS) number as compared to the control extender (CE). EE with MP in the concentrations of 0.2-1.0% v/v significantly reduced ($P \leq 0.05$) the contamination of the semen samples with various microorganisms. The obtained results demonstrated perspectivity of the yeast extracts for effective semen dilution and preservation for further artificial insemination (AI) of animals.

***Key words**: brewer yeast, extender, mannoproteins, microbiological indices, motility indices, preservation, ram semen.*

INTRODUCTION

Artificial insemination (AI) is an essential technique for the development of sheep selection programs. AI with frozen semen is not widely implemented because of the relatively low quality of the latter. The processes of semen freezing and thawing damage ram sperm and reduce the quality of semen (Kulaksiz et al., 2012). Dilution and refrigeration of ram semen can be an alternative to freezing if insemination is done shortly after semen collection (Wusima et al., 2012). The wide use of refrigerated semen for AI is largely due to the possibility of semen dilution (Gundogan et al., 2009). Short term preservation of ram semen with different extenders (Gündogan, 2009). The preservation by refrigeration of ram semen is done at a temperature of 2-4°C and is one of the most intensively used preservation techniques for ovine (Dascăl, 2009). At the same time, the success of this technique is limited by the shortness of time, 24-48 hours, within which the ram sperm maintains sufficient motility and fertilizing capacity (Watson, 2000; Gil et al., 2011).
The main factors affecting the quality of sperm stored by refrigeration are the hypothermic shock, associated with the damage of plasma membranes, the activation of caspases, involved in apoptosis, the DNA hypomethylation and fragmentation leading to decreases of sperm fertilizing capacity, and the oxidative stress, associated with membrane lipid peroxidation (Budai et al., 2014).
The high concentration of polyunsaturated fatty acids in the spermatozoa membranes of ruminants makes them extremely vulnerable to the oxidative stress (Bucak et al., 2010). Reactive oxygen species (ROS) of the H_2O_2, O^{2-} and OH^- type, which are formed during the semen preservation by refrigeration, actively react with unsaturated fatty acids, leading to lipid peroxidation and disruption of mitochondrial and plasma membranes of the ram spermatozoa, affecting the motility of the latter (Amidi et al., 2016). The success of sperm preservation depends on the extender, which must contain substances that protect the spermatozoa from the thermal and oxidative shocks.
Diluting of the ejaculate with an extender allows to obtain seminal material with the

optimal concentration of spermatozoa. At the same time, the extender contains substances with a protective effect and supports the spermatozoa metabolism maintaining their fertilizing capacity. It was found that the extender has to contain substances that promote sperm metabolism and motility, colloidal substances that protect sperm membranes, and buffer substances for maintaining a favorable pH of the medium. These substances are of particular importance for the protective media used for diluting and preserving ram semen, because the high concentration of spermatozoa in the ejaculate and their intense metabolism significantly increase the acidity of the semen preparations (Lopez-Saez et al., 2000; Paulenz et al., 2002; Practical work, 2005).

Usually, the extenders for protection and preservation of the semen are based on skimmed milk, glycerin and egg yolk, which have a cryoprotective effect and help to maintain the quality of ram semen and motility indices for up to 48-96 hours under hypothermic conditions (Gil et al., 2011; Galarza et al., 2019).

Fatty acids, seminal plasma, sugars and various substances with antioxidant activity are used as extender additives to reduce the ROS production in semen preparations during storage, and to improve sperm quality (Toker et al., 2016; Allai et al., 2018). The non-enzymatic antioxidants, known as synthetic antioxidants, such as glutathione, ascorbic acid, citric acid, carotenoids, hypotaurin, ubiquinone and vitamins, are often used for this purpose too (Amidi et al., 2016).

Microbial polysaccharides are also mentioned in the specialized literature as possessing cryoprotective properties. Thus, Zheng et al. found that supplementation of the extender with *Laminaria japonica* water-soluble polysaccharides at different concentrations increased the motility and viability of the bull sperm (Zheng et al., 2017). This effect of polysaccharides from *Laminaria japonica* was due to their high antioxidant activity (Cui et al., 2016).

Sacchamomyces cerevisiae yeasts are recognized as very important sources of antioxidant enzyme systems that include superoxide dismutase and catalase (Lavová & Urminská, 2013). Yeast biomass extracts possess different antioxidant activities, which depend on the method used for their obtaining, and on the contained substances. Usually, the protein and mannoprotein extracts are known for their relatively higher antioxidant activity due to the lateral aromatic chains and free thiols from denatured proteins (Jaehrig et al., 2007; Jaehrig et al., 2008).

Besides that, the extracts from *Saccharomyces* yeast biomass, especially the mannoproteins, are also known for their antimicrobial properties against various pathogenic microorganisms (Ganner et al., 2013; Greco et al., 2018)

In this context, the possibility of obtaining extenders for semen protection based on biologically active substances of microbial origin may present a scientific and practical interest in the matters of the preservation of zootechnically important animals. Therefore, the purpose of this research was to evaluate the influence of MP from brewer yeast on the motility, morphological and microbiological indices of ram semen preserved by refrigeration at 4°C.

MATERIALS AND METHODS

Obtaining the mannoprotein extract

The mannoprotein extract (MP) was obtained from the yeast *Saccharomyces* biomass waste after production of the beer *Lager*. The biomass was offered by the Kellers brewery, the Budești commune, the Chisinau municipality. The semi-liquid microbial waste from the factory was centrifuged at 3500 rpm for 15 minutes to remove the liquid phase. The solid phase, the yeast biomass, was frozen at -18°C for storage. Subsequently, to obtain MP, the biomass was thawed at room temperature and then subjected to autolysis at 45°C for 8 hours, using the sodium phosphate buffer in the 1: 1 ratio. After autolysis, the suspension was centrifuged at 3500 rpm for 15 minutes to remove the liquid phase. The remaining solid residue was hydrolyzed with 1N NaOH solution in the 1: 5 v/v ratio at 80 ± 5°C for 2 hours. After hydrolysis the suspension was centrifuged at 3500 rpm for 15 minutes to separate the liquid phase from the solid phase. The mannoproteins were obtained from the supernatant by sedimentation with the help of the 96% ethyl alcohol, in the ratio of 1:2 v/v, and were repeatedly purified with ethyl alcohol. In this research the MP water solution in the concentration of 500 mg/ml was used.

Collection and selection of semen

The object of the study was the semen from Moldovan Karakul rams, collected by the artificial vagina procedure during the mating season.

The ejaculates of at least 1.0 ml volume, with a sperm concentration of at least 2.5 x 10^9 ml^{-1}, and with the number of motile spermatozoa of at least 70% were selected for the experiments.

Extenders

Two extenders were used for dilution and preservation of the selected ejaculators: 1) the control extender (CE), composed of glucose (0.8%), sodium citrate (2.8%), egg yolk (20%), antibiotics (50000 IU), distilled water (up to 100 ml) (Milovanov et al., 2020), and 2) the experimental extender (EE) without antibiotics, containing sucrose (6.4%), sodium citrate (0.6%), egg yolk (10%), MP in the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1% v/v (0.5-5.0 mg/ml), labeled as EE1, EE2 - EE10.

Motility, speed and morphological indices

Motility and morphological indices of the ram semen: total motility (TM), progressive motility (PM), average path velocity (VAP), straight linear velocity (VSL), curvilinear velocity (VCL) and abnormal sperm (AS) number were analyzed under the phase-contrast microscope with the magnification x400-600, using the CEROS II^{TM} Sperm Analyzer computer program (Hamilton Thorne designs USA).

The semen samples were stored at 4°C. The indices were estimated in the beginning and then at the interval of 24 hours during the following 120-hour period.

Microbiological indices

The determination of microbiological indices in the ram semen, namely the total number of microorganisms (TNM) and the number of colonies of different bacteria and fungi, was carried out in several stages: preparation of successive decimal dilutions (1: 10 - 1: 1000); insemination of 0.1 ml from the dilutions on differential culture media; incubation at +37°C for 24-48 hours and counting the colony forming units (CFU) according to (GOST, 2014). The CFU number was expressed in log CFU/ml.

The Nutrient Agar medium M001 was used for determination of TNM; the Special HiCrome Endo Agar M029R was used for differentiating microorganisms from the *Enterobacteriaceae* family; HiCrome ECC Agar M1293 - for *E. coli*, *Ps. aeruginosa*; HiCrome Bacillus Agar Base M1651-for *Bacillus* spp.; Anaerobic Agar M228 - for *Clostridium* spp.; *Streptococcus* Selection Agar M304 - for *Staphylococcus* spp.; Sabouraud Dextrose Agar M063 - for yeast.

The identification of the bacterial genuses was performed based on the morphocultural, morphotinctorial properties and on the fermentation capacities in relation to some carbohydrates (glucose, dextrose, fructose, maltose, etc.) (The Shorter Bergey's Manual of Determinative Bacteriology, 1980)

The semen microbiological indices were determined after 120 hours of sample storage at the temperature of 4°C.

Statistical indices

The experiments were performed in five replicas. The obtained data were analyzed statistically and the results were expressed as the mean ± SEM. Student's t-test was done using the Microsoft Excel software. Differences with P values ≤ 0.05 were considered as statistically significan

RESULTS AND DISCUSSIONS

The ram semen preservation method by refrigeration is based on the use of protective media for diluting and maintaining the quality of semen for a maximum period of time (for maintaining the motility and fertilizing capacity of spermatozoa) while stored at 4°C.

The sperm motility test is usually used for evaluations of the effectiveness of the protective media (Věžník et al., 2004).

The sperm motility, especially the progressive sperm motility, is the main indicator of sperm quality and the determining factor of animal fertility, including for sheep (David et al., 2015; Hering et al., 2014; Simon et al., 2011).

The total motility (TM) of the ram sperm in semen preserved by refrigeration on EC and EE for 120 hours at 4°C is shown in Table 1. The initial TM for CE was 87.8% and then it constantly decreased within the 120 hours of storage reaching 58.3% by the end (Table 1). For the EE4-EE10 range, TM was from the very beginning significantly higher ($P \leq 0.05$) than for CE (90.2-92.5%), and the decrease rate during storage was smaller. Thus, comparing to CE there were observed statistically significant differences ($P \leq 0.05$) after 24 hours of storage for EE4-EE8, after 48 hours - for EE2-EE9, and after 72 hours - for EE3, EE5 and EE7 (Table 1). After 96 hours of storage TM was significantly higher ($P \leq 0.05$) for EE6 - EE10 - respectively, 71.0-77% comparing to 63% in the CE variant (Table 1). There were also observed significant TM differences ($P \leq 0.05$) between some EE variants and EE1. Thus, comparing to EE1, statistically higher TM values ($P \leq 0.05$) were observed after 0, 24, 48, 72 and 96 hours of storage for the same range of MP concentrations in the extender (Table 1). After 120 hours of storage TM for EE2-EE10 was still higher than in CE and EE1, but the differences were not significant statistically ($P > 0.05$) (Table 1).

Table 1. Total Motility of ram sperm (%) in the semen preserved by refrigeration within 120 hours of storage at 4°C

Extender	**Conservation time, hours**					
	0	24	48	72	96	120
CE	87.8±0.7	83.8±2.0	81.2±1.0	71.2±3.9	63.0±2.5	58.3±2.0
EE1	87.8±1.0	83.8±2.0	82.0±1.4	78.5±1.7	65.3±1.8	57.3±2.7
EE2	88.2±0.7	87.6±1.4	84.2±1.0^{a}	74.0±4.5	69.3±3.9	61.8±5.3
EE3	88.8±1.7	86.5±1.3	85.3±1.2^{a}	80.3±0.9^{a}	70.5±3.9	62.5±4.9
EE4	90.2±0.7^{a,b}	88.8±1.7^{a,b}	86.6±0.7^{a,b}	77.8±4.2	71.0±3.8	63.0±5.1
EE5	91.0±1.1^{a,b}	90.5±1.2^{a,b}	86.8±0.9^{a,b}	82.5±1.0^{a,b}	72.8±3.6	63.0±5.3
EE6	91.8±0.8^{a,b}	91.0±1.1^{a,b}	86.8±1.2^{a,b}	77.2±5.6	75.3±4.9^{a,b}	66.3±5.8
EE7	92.5±1.3^{a,b}	91.8±1.1^{a,b}	88.5±1.3^{ab}	82.5±1.0^{a,b}	76.3±3.2^{a,b}	67.5±4.6
EE8	92.0±0.9^{a,b}	88.4±1.3^{a,b}	87.0±1.1^{a,b}	78.2±3.1	77.0±3.5^{a,b}	68.0±5.8
EE9	91.3±0.6^{a,b}	88.5±1.7	85.5±1.3^{a}	79.0±1.6	74.0±3.3^{a,b}	65.0±5.4
EE10	90.8±0.7^{a,b}	85.6±2.2	83.2±1.4	75.0±3.7	71.0±1.8^{a,b}	62.3±4.8

^{a} - statistically significant differences ($P \leq 0.05$) comparing to CE; ^{b} - statistically significant differences ($P \leq 0.05$) comparing to EE1

The progressive motility (PM) of the ram sperm in the semen preserved by refrigeration with CE and EE within 120 hours of storage at 4°C is shown in Table 2.

As in the case of TM, PM of the ram sperm constantly decreased with time regardless of the extender composition (Table 2). The application of MP in the extender affected PM too, but the changes were not as obvious as in the case of TM. Thus, statistically significant differences ($P \leq 0.05$) comparing to CE and EE1 were registered after 24 hours only for EE4 and EE6, and after 96-120 hours - only for EE8 (Table 2). In the other cases no significant differences were observed ($P > 0.05$) (Table 2).

Table 2. Progressive Motility of ram sperm (%) in the semen preserved by refrigeration within 120 hours of storage at 4°C

Extender	**Conservation time, hours**					
	0	24	48	72	96	120
CE	45.2±4.8	36.8±2.1	38.6±5.2	35.2±3.9	26.0±4.1	12.8±1.1
EE1	50.3±3.2	33.0±3.9	39.8±5.9	36.3±5.8	24.8±3.3	14.3±2.9
EE2	51.2±1.4	42.2±4.1	39.8±4.4	32.0±5.9	28.8±4.8	15.5±5.3
EE3	50.5±1.6	38.0±6.6	37.8±4.0	38.0±4.4	24.8±3.9	15.5±5.6
EE4	42.8±2.6	46.2±4.4$^{a, b}$	34.8±1.7	37.0±7.2	33.0±8.4	19.0±4.8
EE5	46.5±3.6	40.8±3.8	33.0±2.2	29.5±3.7	26.8±4.8	17.0±4.7
EE6	51.8±2.9	47.0±3.9$^{a, b}$	43.6±4.6	38.8±9.4	33.0±5.4	18.5±6.0
EE7	51.8±2.2	45.8±5.3^{b}	42.8±3.1	36.5±7.3	32.8±7.5	22.3±7.6
EE8	48.0±2.2	39.6±3.9	38.6±3.8	33.6±6.6	38.5±4.9$^{a, b}$	25.5±4.7$^{a, b}$
EE9	51.3±6.3	37.3±5.6	45.8±5.3	42.8±5.8	30.8±5.9	17.8±3.0
EE10	46.4±3.5	39.6±5.6	40.0±5.5	30.0±6.4	31.8±6.7	14.8±2.6

^{a} - statistically significant differences ($P \leq 0.05$) comparing to CE; ^{b} - statistically significant differences ($P \leq 0.05$) comparing to EE1

The data on VAP, VSL and VCL indices of the ram sperm in the semen preserved by refrigeration are shown in Table 3.
According to the obtained results, the values of these indices continuously decreased within 120 hours of storage (Table 3). There were observed no significant differences in VAP, VSL and VCL values between all the variants after 0, 24, 48 and 72 hours of storage, (Table 3). However, significant differences in VAP and VSL values did appear between CE and EE8 after 96 hours. Although after 120 hours the VAP and VSL values for EE8 were still higher than for CE, the differences were not significant statistically (Table 3).

Table 3. VAP, VSL, VCL (µm/s) of the ram sperm in the semen preserved by refrigeration within 120 hours of storage at 4°C

Conserv. time, hours	**Indices, µm/s**	**CE**	**EE1**	**EE2**	**EE3**	**EE4**	**EE5**	**EE6**	**EE7**	**EE8**	**EE9**	**EE10**
0	**VAP**	132.9±5.9	135.3±3.2	135.7±6.4	139.5±5.5	118.5±6.8	125.4±6.9	133.1±6.7	136.6±2.7	130.9±2.8	133.3±4.1	126.4±5.3
	VSL	109.9±6.7	113.8±4.0	114.0±5.2	117.1±5.1	94.6±5.3	103.8±5.9	110.4±5.6	112.1±3.9	106.6±2.5	112.0±5.7	106.4±7.6
	VCL	192.8±9.4	190.7±1.9	190.3±11.5	203.4±5.2	179.2±14.9	184.0±13.3	191.0±10.0	205.4±7.2	196.6±7.6	193.8±6.3	186.1±8.7
24	**VAP**	106.9±6.5	105.7±5.2	101.7±4.3	109.3±7.2	115.2±6.6	111.0±1.5	113.0±3.3	111.3±5.2	102.9±6.0	98.6±5.1	98.7±5.6
	VSL	84.3±6.5	78.1±2.4	80.5±5.3	83.8±7.5	92.0±6.7	85.0±3.0	89.2±3.2	89.2±5.8	80.4±3.9	76.8±5.2	80.0±5.7
	VCL	172.4±9.1	178.8±10.4	168.4±7.2	180.2±12.7	183.5±11.4	185.1±5.0	183.6±9.9	179.2±11.0	167.0±13.3	162.0±11.6	156.7±9.0
48	**VAP**	104.6±5.7	108.9±2.7	102.6±3.7	100.4±4.9	96.7±7.1	100.1±5.5	108.3±4.5	110.0±6.0	99.9±5.1	113.3±5.2	106.9±6.9
	VSL	85.7±7.2	86.2±4.6	79.7±3.9	80.1±5.2	71.8±1.9	75.9±4.6	83.2±4.3	82.4±4.2	78.5±4.2	91.2±6.5	86.0±6.8
	VCL	166.9±7.9	177.1±4.4	165.7±8.1	167.0±7.4	150.3±7.6	169.4±10.1	156.3±8.9	168.8±10.1	159.2±8.5	172.6±6.6	171.4±10.9
72	**VAP**	100.5±9.7	102.0±7.3	88.9±6.9	98.4±4.5	94.1±7.8	92.0±3.2	94.3±12.0	99.6±8.3	85.7±4.3	98.6±8.4	85.3±6.2
	VSL	82.3±9.5	83.2±7.9	71.0±6.8	80.4±5.9	77.2±7.9	69.7±3.7	81.6±9.8	78.8±9.3	68.3±4.6	81.9±9.1	67.8±6.7
	VCL	160.1±9.8	166.4±8.3	146.2±9.5	158.1±3.6	152.9±11.1	146.9±9.2	156.9±15.5	161.3±7.2	138.5±6.1	159.0±7.4	132.5±6.6
96	**VAP**	80.7±4.6	85.8±5.8	85.9±2.7	82.7±7.0	87.7±8.0	86.8±4.9	93.9±6.8	90.3±5.9	97.8±5.2[a]	93.3±6.5	94.7±7.8
	VSL	57.9±4.1	67.8±5.3	65.5±3.1	66.4±5.8	73.0±8.1	67.0±6.0	76.3±9.1	71.5±8.1	78.7±6.2[a]	74.6±9.1	76.4±9.7
	VCL	145.1±8.1	147.6±6.2	150.5±2.5	144.6±11.8	142.7±10.3	155.0±8.0	156.8±5.1	155.1±5.1	158.9±4.8	154.1±4.8	158.8±5.0
120	**VAP**	73.1±2.0	76.1±1.5	72.7±7.9	74.6±6.7	79.2±6.2	74.1±4.8	76.7±5.4	76.4±6.9	80.3±9.4	72.4±3.6	71.2±2.7
	VSL	54.8±0.6	55.4±3.9	57.0±5.2	52.0±3.6	58.5±5.2	56.1±5.6	56.6±6.6	58.2±7.5	64.4±9.0	52.9±3.4	52.8±2.7
	VCL	137.6±6.1	136.0±6.7	125.0±14.6	131.2±12.1	135.4±11.9	132.0±6.4	133.0±7.6	132.0±11.3	136.7±12.9	126.3±6.9	125.0±6.0

[a] - statistically significant differences (P≤0.05) comparing to CE

Since the number of spermatozoa with various morphological anomalies (abnormal sperm AS) usually increases with storage, this parameter was monitored within 120 hours for EE with MP in the concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0% v/v, as well as for CE (Table 4).
AS increased constantly during storage at +4°C in all variants (Table 4). In the case of CE AS initially was 11.0% and increased up to 17.7-18.8% after 96 and 120 hours of storage, respectively (Table 4). As for the EE variants, AS in the beginning varied within 8.9-10.4%, and increased to 15.7-17.3% after 96 hours, and to 16.8-18.4% after 120 hours of storage (Table 4).

Table 4. Abnormal Sperm (%) in the semen preserved by refrigeration within 120 hours of storage at 4°C

Extender	Conservation time, hours					
	0	24	48	72	96	120
CE	11.0±0.6	12.9±2.1	16.1±3.4	16.7±2.3	17.7±1.8	18.8±1.4
EE2	10.4±1.6	12.3±1.7	16.2±1.7	16.2±2.5	17.3±1.7	18.4±1.5
EE4	9.6±1.7	11.9±1.7	15.9±1.5	16.0±2.4	17.4±2.0	18.0±1.4
EE6	8.9±1.8	11.7±1.7	15.7±2.7	15.2±2.1	16.7±1.5	18.3±2.4
EE8	8.9±1.1	12.3±1.8	15.3±2.1	15.2±2.0	15.7±2.0	16.8±1.2
EE10	9.2±1.3	12.8±1.4	16.4±2.4	15.7±1.9	15.7±1.5	17.9±2.4

Because collection and processing of ejaculates is not done under strictly sterile conditions, usually there is microbial contamination in the samples, that can have a negative effect on the quality of the semen.

The microbiological indices (the total number of microorganisms and the number of colonies from different taxonomic groups by the end of 120 hours of storage at 4°C) were determined for the samples with the range of MP concentrations similar to that used for AS. The results are shown in Table 5.

The microbiological profiles in the EE samples were quite similar to that in the CE sample, but they differed quantitatively. Thus, by the end of 120 hours of storage in all EE samples both TNM and the number of colonies of microorganisms from different taxonomic groups were significantly lower ($P \leq 0.05$) comparing to those for CE. The only exception was the EE2 case, where the number of *Escherichia coli* colonies practically did not differ from the control (Table 5).

Significant differences ($P \leq 0.05$) were also found between the number of microorganisms in the EE4-EE10 cases and EE2, which had the smallest concentration of MP (Table 5). It should also be noted that bacteria of the *Clostridium* genus and fungi in general were especially sensitive to the presence of MP in the extender. The use of EE8 and EE10 resulted in total inhibition of *Clostridium* spp.*,* while the minimal concentration of MP in EE2 significantly decreased the number of yeasts colonies with 2.2 log CFU/ml (comparing to the CE case). Also, the use of EE8 and EE10 reduced TNM with 1.9-2.7 log CFU/ml, *Escherichia coli* - with 1.0-2.2 log CFU/ml and *Bacillus* spp. - with 1.8-2.4 log CFU/ml (Table 5). *P. aeruginosa, Salmonella* spp. and *Staphylococcus* spp. were not detected in any of the investigated samples (Table 5).

Table 5. The microbiological indices of the ram semen after 120 hours of storage at 4°C

Microrganisms	Colonies, log CFU/ml					
	CE	EE2	EE4	EE6	EE8	EE10
TNM	5.8±0.08	5.4±0.12[a]	5.1±0.14[a,b]	5.1±0.11[a,b]	3.9±0.03[a,b]	3.1±0.05[a,b]
Escherichia coli	5.1±0.19	5.1±0.11	4.5±0.06[a,b]	4.5±0.06[a,b]	4.1±0.17[a,b]	2.9±0.02[a,b]
Clostridium spp.	5.5±0.08	5.0±0.03[a]	4.9±0.03[a]	4.7±0.05[a,b]	n.d.*	n.d.*
Bacillus spp.	5.3±0.09	4.9±0.05[a]	4.9±0.03[a]	4.1±0.12[a,b]	3.5±0.06[a,b]	2.9±0.02[a,b]
Yeast	5.5±0.06	3.3±0.08[a]	2.8±0.05[a,b]	2.8±0.04[a,b]	2.8±0.03[a,b]	2.7±0.04[a,b]
Staphylococcus spp.	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d*
Salmonella spp.	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*
Pseudomonas aeruginosa	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*

[a] - statistically significant differences ($P \leq 0.05$) comparing to CE; [b] - statistically significant differences ($P \leq 0.05$) comparing to EE2; n.d.* - not detected

The TM, PM, VAP, VSL and VCL indices were constantly declining, while AS was increasing during the semen storage, regardless of the used extender medium and the initial values of these indices (Hegedűšova et al., 2012; Moustafa et al., 2015).

SOD, CAT, glutathione peroxidase (GPx) and glutathione reductase (GR) are known as the major antioxidant enzymes involved in self-protection of mammalian spermatozoa from reactive oxygen species (ROS) (Bansal et al., 2011). These enzymesboth reduce ROS excesses and prevent spermatozoa damage. For example, SOD inactivates the superoxide ion (O^{2-}) - the main cause of ROS production, transforming it into hydrogen peroxide (H_2O_2), that subsequently is transformed into oxygen (O_2) and water (H_2O) by CAT.

Amino acids also have antioxidant properties. Being non-enzymatic antioxidants, they are present in significant quantities in the seminal plasma. It is known that supplementing extenders with various amino acids, such as taurine, hypotaurine, proline, glutamine, glycine, histidine and cysteine, reduces DNA fragmentation, increases the motility, viability and fertility of the ram sperm (Bucak et al., 2009; Sarıozkan et al., 2009; Bucak et al., 2013).

Cysteine and glutathione maintain the integrity of the acrosome, spermatozoa membrane, increase sperm quality and motility after cryopreservation (Bilodeau et al., 2001; Coyan et al., 2011; Sharafi et al., 2015).

Methionine is another amino acid that can be used to preserve the ram sperm by refrigeration. The extender supplementation with 1, 2 and 4 mM of methionine increased motility, viability and mitochondrial activity of ram spermatozoa (Bucak et al., 2012).

Roostaei-Ali Mehr & Noori (2013) established that sperm motility, viability, and membrane integrity could be increased by supplementing the protective medium for cryopreservation of the ram semen with 40- 80 mM of L-glutamine.

Bovine serum albumin (BSA) had a similar effect, protecting the ram sperm during the freeze-thaw processes (Uysal et al., 2007). At the concentrations of 10% or 15% in the protective medium BSA could be used for cryopreservation as a substitute for the egg yolk (Matsuoka et al., 2006).

Mono- and disaccharides are also widely used for the ram semen storage. Carbohydrates serve as an energy substrate for spermatozoa during preservation, they maintain osmotic pressure and offer fluidity to sperm cell membranes (Aboagla et al., 2003). Supplementing the extenders with different carbohydrates in various concentrations increased the motility, viability, membrane and acrosome integrity of the ram sperm, and reduced the number of spermatozoa with various anomalies (Ahmad et al., 2015; Jafaroghli et al., 2011).

Protective substances of various kinds may be and are included in the extenders for the efficient semen preservation (Larbi et al., 2018), but it should be noted that when used in excessive quantities they can also cause biochemical and functional damage, and modify the spermatozoa morphology. Thus, according to Câmara et al. (2011), supplementation of the extender with glutathione or catalase in the concentration of 400 mM and 400 U/mL, respectively, was toxic for spermatozoa and negatively affected the ram sperm motility.

Based on the above, the positive effect of EE on the motility and morphological indices of the ram spermatozoa was due to the biochemical composition and activity of the antioxidant enzymes CAT and SOD of MP. MP contained 36.6±0.58% S.U. of protein covering the full range of the essential and non-essential amino acids, and 40.9±3.04% S.U. of carbohydrates. It had a total antioxidant activity of 29.1±1.5 mg trolox/g S.U., and the activity of its antioxidant enzymes CAT and SOD was 741.2±44.8 mmol/min per mg protein and 66.2±2.9 U/mg protein, respectively (Chiselița et al., 2022).

The use of EE with different concentrations of MP for diluting the ram semen positively influenced the total motility (TM) and progressive motility (PM) of the ram sperm within 96 hours of storage, and did not have negative effects on these parameters after 120 hours of storage. Thus, TM (71.0-77.0%) in the EE6-EE10 experimental samples (Table 1) and PM (38.5%) in the EE8 sample (Table 2) were significantly higher ($P \leq 0.05$) than both in the CE and EE1 variants after 96 hours of storage. This proves that the improvement of these indices comparing to the control, was caused by the supplementation of the extender with MP.

Considering the fact that significant differences ($P \leq 0.05$) between the CE and EE8 variants after 96 hours of storage were observed only for VAP and VSL (Table 3), we can conclude that the extender supplementation with MP had a relatively small impact on the VAP, VSL and VCL indices. At the same time, the absence of statistically significant differences for these indices between all the EE variants and the CE at different storage periods can be considered as a positive factor, since it implies that the supplementation did not have negative effects on the VAP, VSL and VCL indices of the ram spermatozoa.

Extender supplementation also had statistically insignificant impacts on AS. Thus, the minimum AS was observed for EE8 and EE10, reaching 15.7% after 96 hours of storage, and 16.8-17.9% after 120 hours, and that was 11.3% and, respectively 4.8-10.6% less comparing to CE (Table 4).

The results of our research show that in the matters of ram semen preservation by refrigeration the extender supplemented with different concentrations of MP was either as efficient or even better than the other extenders including the commercial ones (Hegedűšova et al., 2012; Maksimović et al., 2018).

According to the experts in the field, the rate of microbiological contamination of the ejaculates oscillates between 43 and 100%. This microbiological contamination, among other endogenous and exogenous factors, can contribute to decreases of the semen qualityand fertility. Microbiological contamination of the diluted semen causes low spermatozoa viability, decreased conception rate and reduced offspring number (Jäkel et al., 2021). For example, high bacteria abundance in porcine sperm decreased the sperm motility and viability, and increased the percentage of spermatozoa with various defects (Kuster et al., 2016).

Among the main causes of bacteriospermia are poor hygienic conditions in profile companies, especially during semen collection, contaminated water, feed and air, as well as exposure to hair, skin, respiratory secretions or fecal masses (Althouse et al., 2000).

To solve the problems associated with this microbiological contamination and to correspond to Council Directive 90/429/EEC, Annex C2 of European Union (Council Directive, UE), the extenders intended for the semen preservation are usually supplemented with various antibiotics (Althouse et al., 2008; Tvrdá et al., 2021). However, the continued use of antibiotics facilitates appearance, spread and persistence of multidrug-resistant bacteria (Morrell et al., 2014).

According to Tvrdá et al. (2022), the ram semen was contaminated by bacteria of the genuses *Staphylococcus*, *Escherichia*, *Bacillus*, *Pseudomonas*, *Lactobacillus*, *Acinetobacter*, *Aeromonas*, *Enterobacter* etc.

The microbiological indices of the ram semen obtained after 120 hours of storage demonstrated that the supplementation of the extender with MP significantly influenced the number of microorganisms in the samples. Thus, the use of EE8 and EE10 reduced TNM with 1.9-2.7 log CFU/ml, *Escherichia coli* - with 1.0-2.2 log CFU/ml, and *Bacillus* spp. - with 1.8-2.4 log CFU/ml (Table 5). *Clostridium* genus bacteria and yeast in general were even more sensitive to the presence of MP in the extender. The use of EE8 and EE10 resulted in total inhibition of *Clostridium* spp., and even the minimal concentrations of MP (EE2, Table 5) significantly decreased the number of yeasts colonies with 2.2 log CFU/ml comparing to CE. The significant decreases in the numbers of microorganisms observed between EE2-EE10 and CE on the one hand, and between EE4-EE10 and EE2 on the other is evidence that the supplementation of the extender with MP had a decontaminating effect on the samples that was proportional to the applied MP concentrations (0.2-1.0% v/v).

The mannoprotein extracts are known for their antibacterial activity against *Escherichia coli, Salmonella* spp. and some fungi (Posadas et al., 2010; Trevisi et al., 2012). Santovito E. et al. also found that the yeast mannoprotein preparations inhibited *in vitro* the growth of various *Clostridium perfringens* strains. The inhibitory effect depended on the yeast strain used for mannoprotein extraction and on the dose. The mannoprotein extract from baking yeasts proved to be the most effective. Its minimal inhibition concentration of 1.25 mg/ml increased the time of the *lag* phase with 3.6 hours, reduced the maximum growth rate by more than 50%, and reduced the colony number with 10^2 CFU/ml in 24 hours, comparing to the control (Santovito et al., 2019).

Moreover, a recent *in vivo* research revealed a beneficial effect of the yeast mannoprotein products for maintaining the performance and health of birds, minimizing mortality from infections caused by *Clostridium perfringens* without any use of antibiotics (Fowler et al., 2015; Hashim et al., 2018).

Various natural biologically active extracts from plants, oilseeds, herbs, fruits, and vegetables are discussed in the specialized literature as potential supplements for dilution, preservation and storage of the animal semen (Del Valle et al., 2013; Baghshahi et al., 2014;

Motlagh et al., 2014; Larbi A et al., 2016; Ros-Santaella et al., 2021).

In this context, considering the presented results and the fact that MP was obtained from the yeast biomass from beer industry waste, we can suggest that biologically active yeast extracts can be used as an effective alternative to the supplements mentioned above.

CONCLUSIONS

EE supplemented with MP in the concentrations of 0.6-0.9% v/v significantly increased ($P \leq 0.05$) the total motility (TM) and the progressive motility (PM) of the ram spermatozoa during 96 hours of storage, and did not affect negatively the VAP, VSL and VCL indices. The MP concentrations of 0.8-1.0% v/v reduced the number of abnormal sperm (AS) comparing to CE after 96-120 hours of storage. The supplementation of EE with MP in the concentrations of 0.2-1.0% v/v significantly reduced ($P \leq 0.05$) the contamination of the semen samples with various microorganisms. The obtained results demonstrated the perspectivity of the yeast extracts for diversification of natural biologically active supplements, designed for effective semen dilution and presservation for artificial insemination (AI) of sheep.

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REPRODUCTIVE QUALITIES OF DAIRY COWS AT DIFFERENT AGE AND LEVELS OF MILK YIELD

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Abstract

The study results of the reproductive ability of cows depending of their age and milk productivity are presented. The studies were conducted in the Republic of Moldova on cows of the Holstein breed. The optimal parameters for the duration of the SP and CI were detected in cows with milk productivity less than 6000 kg of milk, at which the animals were characterized by the maximum indicators of calves yield and CCR. In cows with a milk yield of 9001 kg or more, a significant increase in the duration of SP (+109.8 days) and the CI (+90.3 days) was observed, the CCR decreased by 0.22, and the yield of calves/100cows per years by 21.7 heads. On average, each increase in milk yield by 1000 kg increases the duration of the SP by 27.6 days, the CI by 20.9 days. The share of the impact (η^2_x) of level of milk yield on the duration of SP was 18.0% ($p<0.001$), on the duration of the CI 15.6% ($p<0.001$), on the CCR 25.6% ($p<0.001$), the yield of calves 35.6 % ($p<0.001$). In order to increase the economic profit, we recommend livestock holdings to monitor the duration of SP.

***Key words**: correlation, dispersion analysis, Holstein breed cows, level of milk productivity, reproductive capacity.*

INTRODUCTION

Cattle reproduction is one of the most laborious processes in breeding and exploitation of dairy cattle. The productivity of milk in cows, the efficiency of selection and reproduction work, the duration and intensity of the use of animals with highly productive genetic value depend on the level of reproduction of the herd.

In the modern conditions of market relations, the primary task in the field of dairy cattle breeding is the breeding of economically profitable animals. The female stock must have a high productivity potential but also a high level of reproductive performance in order to obtain as many young breeding animals as possible. In recent years, in the dairy cattle breeding, along with the increase in the genetic potential of milk productivity, a tendency to decrease the reproduction function of cows has been observed (Gabor, 2008; Popescu, 2014; Firsova et al., 2017; Aminova et al., 2019; Vasil'eva, 2019). Which leads to a reduction in the period of their exploitation period, a decrease in the level of productivity, and, consequently, the profitability of the branch as a whole (Dunin et al., 2019).

The state of the reproductive function of cows depends on many factors: heredity, artificial insemination technology, conditions of maintenance, feeding, exploitation, physiological state of the animal, etc. (Tanana & Peshko, 2011; Agalakova & Tyapughin, 2013; Berezkina et al., 2019; Meshcherov et al., 2019). According to a number of scientific studies increase in the level of milk productivity leads to decrease in the fertility of animals, as a result the length of the service period increases but the coefficient of reproductive capacity and the yield of calves per 100 cows per year decreases (Saksa & Barsukova, 2007; Gromova et al., 2016; Revina & Astashenkova 2018). Marusich, (2017) believes that the cause of the antagonism between productivity and reproductive function is primarily unilateral selection aimed at obtaining high yields, but not taking into account factors affecting health and reproductive function. Therefore, parallel to an increase in milk productivity, a primordial feature, from an economic point of view, another equally important task is taking shape, such as improving the reproductive abilities of the cows (Golikova & Fedoseeva, 2016).

Researchers and practitioners in many countries report the influence of milk yield on the reproductive function of cows and the antagonistic relationship between the milk productivity level of cows and their fertility (Sangsritavong, et al. 2002; Wiltbank et al. 2006; Kononov, 2013).

But there are also contradictory data (Bolgov & Karamonova, 2003; Sudarev, 2008) which did not motivate the researchers to reach a consensus.

The purpose of the research - the study of reproductive indices in Holstein cows according to age and the level of milk productivity, and the interrelationships between breeding indicators and milk productivity indices.

MATERIALS AND METHODS

In the research was the population of Holstein breed cows from the SRL "Holstein" livestock farm, Roshkani village, Anenii Noi District. The material for the research was the data of zootechnical accounting (card form T-2) and the information database of the farms. The processing included data on milk productivity and reproductive performances of Holstein breed cows. Milk productivity was estimated by milk yield for 305 days of lactation and for full lactation, taking into account milking days. The reproductive indices was studied by the duration of the service period (SP) and calving interval (CI), the coefficient of reproductive capacity (CCR), and the number of calves obtained per 100 cows/year (birth rate – BC).

The coefficient of reproductive capacity (CCR) of the female population was calculated according to Èisner et al. (1978).

The birth rate was calculated according to the relationship (Marusich, 2017):

$$\mathrm{BR} = \frac{365 - SP}{285} * (100 - \mathrm{C}) \ldots\ldots\ldots\ldots\ldots (1),$$

where: BR - birth rate (%).

365 - the number of days in a year.

SP - duration of the service period.

285 - the average length of gestation.

C - correction coefficient, taking into account abortions, stillborn calves, etc. (about 2-5%).

For establish the impact of milk productivity on indices of the reproductive function of cows, they were divided into five groups depending on the level of milk yield for 305 days and for the full lactation with a class interval of 1000 kg; ≤ 6000 kg or less; 6001-7000 kg; 7001-8000 kg; 8001-9000 kg; ≥ 9001 kg and more.

The studies were carried out by comparing groups of animals in terms of reproductive abilities at different levels of productivity. Correlation and regression analyzes were used to search for and obtain objective results reflecting the relationship between milk yield and reproductive traits that meet the objectives of dairy cattle selection and breeding activity.

The influence of the milk productivity on the reproductive indices was determined by one-factor variance analysis. The reliability of the difference between the mean values of the signs was determined by Student's td-test.

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-2007 analysis software package.

RESULTS AND DISCUSSIONS

Reproductive capacity is an important component of dairy cattle breeding technology. Sudarev et al. (2015) claim that annual calving contributes to profitable milk production, and regular obtaining of calves in sufficient numbers makes it possible to carry out selection and breeding work at a high level and serves as a basis for extensive reproduction of the herd and, consequently, increases the branch's profitability. In Table 1 are presented the characteristics of cows according to the level of milk and the characteristics of the reproductive capacity of cows in the dynamics of lactations. The analysis of the obtained data showed that with age, milk yield increases in cows, while the duration of the service and calving interval increases. The shortest service period for first-calf heifers was 157.6 days.

Table 1. Milk productivity and reproductive qualities of Holstein cows in the dynamic of lactations, $X \pm Sx$

Indicators	Lactation				
	I	II	III	IV	V
Number of heads	394	165	78	105	64
Milk yield for 305 days, kg	6649.0±70.6***	7718.5±123.7***	7807.4±148.5***	8173.8±119.8***	8082.3±51.8**
Number of milking days	369.6±6.5	356.8±6.5	332.0±10.3**	354.0±7.3	404.9±5.0
Milk yield for full lactation, kg	7028.3±96.4	8355.3±171.8	8910.5±277.9	9326.4±256.4	9162.0±202.5
Service period, days	157.6±5.8	161.1±6.5**	169.8±10.5***	186.9±7.1***	199.8±4.8***
Calvings interval, days	404.5±4.7	424.2±5.5	460.2±10.1	422.5±6.7	431.7±5.0
Reproductive capacity coefficient (CCR)	0.90±0.01	0.88 ±0.01***	0.81±0.02***	0.89±0.01***	0.84±0.01***

**P<0.01. ***P<0.001.

With an increase in milk yield for 305 days of second lactation by 1069.5 kg (16.1%), the duration of the service period increased by 3.5 days (2.2 %). The length of the calving interval was the most optimal in first-calving heifers (404.5 days), but with the increase in milk production for normal lactation (305 days), it increased by 19.7 days (4.9%).

The coefficient for first lactation was 60.2%, while for the calving interval was 16.5%. With the advancing age of the cows, the variability of these indices decreases, reaching in cows in the V^{-th} lactation 32.2% and 10.45%, respectively. The influence (η^2_x) of milk production on the variability of the duration of the service period and the calving interval was at the level of 51.1%) and (28.4%) ($p<0.01$).

The further increase in milk productivity during normal but also full lactation can be seen up to the fourth lactation, after which it decreases in cows in the fifth lactation for a statistically significant difference compared to animals from II^{-th} and I^{-st} lactation. At the same time, the duration of the service period is on the rise, in the dynamics of lactations, and reaches its maximum value in animals in the fifth lactation.

The coefficient of reproductive ability characterizes the fertility of the breeding stock of cattle, depends on the duration of the calving interval, and at the optimal level of fertility of cows is equal to one (Titova & Zabiyakin, 2020). In the case analyzed by us, this indicator reached the highest value in animals of the first lactation, while the minimum indicator was registered in the group of cows in the III lactation with a statistically significant difference.

Between lactation for 305 days, full lactation and duration of service and calving period, the presence of positive statistically significant correlation ($p<0.001$) are established (Table 2).

Table 2. Relationship between the breeding indices and milk yield of Holstein breed cows in lactations dynamic

Indicators	Correlation coefficient, r				
	I lactation	II lactation	III lactation	IV lactation	V lactation
Service period					
Milk yield/305 days of lactation, kg	0.38±0.06***	0.09±0.08	0.69±0.06***	0.64±0.06***	0.69±0.07***
Overall fat/305 days of lactation, kg	0.37±0.06***	0.08±0.08	0.66±0.06***	0.65±0.06***	0.69±0.07***
Milk yield/full lactation, kg	0.24±0.07***	0.08±0.08	0.54±0.08***	0.42±0.08***	0.50±0.09***
Overall fat/full lactation, kg	0.28±0.07 **	- 0.03±0.08	0.55±0.08 ***	0.41±0.08***	0.30±0.12**
Calvings interval					
Milk yield/305 days of lactation, kg	0.38±0.06***	0.10±0.08	0.69±0.06***	0.64±0.06***	0.62±0.08***
Overall fat/305 days of lactation, kg	0.37±0.06***	0.09±0.08	0.67±0.06***	0.65±0.06***	0.62±0.08***
Milk yield/full lactation, kg	0.13±0.07	- 0.13±0.08	0.51±0.08***	0.27±0.09**	0.46±0.08***
Overall fat/full lactation, kg	0.19±0.07**	- 0.08±0.08	0.52±0.08***	0.28±0.09**	0.28±0.1**
Reproductive capacity coefficient					
Milk yield/305 days of lactation, kg	- 0.37±0.06***	- 0.04±0.08	- 0.66±0.06	- 0.58±0.06***	0.69±0.07***
Overall fat/305 days of lactation, kg	- 0.36±0.06***	- 0.03±0.08	- 0.64±0.07***	- 0.58±0.06***	0.49±0.08***
Milk yield/full lactation, kg	- 0.16±0.07*	0.19±0.08 *	- 0.52±0.08***	- 0.30±0.09**	- 0.40±0.11**
Overall fat/full lactation, kg	- 0.23±0.07**	0.09±0.08	- 0.53±0.08***	- 0.31±0.09***	- 0.24±0.12*

*p<0.05; **p<0.01; ***p<0.001.

Similar results were also highlighted regarding the interrelationships between overall fat on normal and total lactation ($p<0.0$; $p<0.001$). Regarding lactation and overall fat for 305 days, full lactation yield and coefficient of reproductive ability of cows was established the presence of negative statistically significant correlation ($p<0.05$; $p<0.0$; $p<0.001$).

The data, presented in Table 3, characterized the changes in the signs of reproductive ability of first heifers depending on the level of milk for 305 days of the first lactation.

Table 3. Reproductive qualities of first lactation Holstein cows depending on the level of milk production (X ± Sx)

Group	Graduation by milk yield, kg	n	I-st lactation					
			Milk yield for 305 days, kg	Service period, days	Milk yield for the full lactation, kg	Calving interval, days	Birth coefficient, %	CCR
1	≤ 6000	26	5467.4±151.3	87.8±20.0	5644.1±277.8	354.3±15.5	98.4±7.5	1.03±0.04
2	6001-7000	56	6614.5±45.2	124.5±14.1	7104.5±141.1	388.8±11.0	93.2±4.6	0.96±0.02
3	7001-8000	114	7502.0±39.4	136.2±9.7***	8616.5±225.2	412.6±8.7***	84.1±2.4	0.87±0.02***
4	8001-9000	110	8408.8±33.1	180.8±9.7***	10210.0±224.1	428.1±8.0***	77.9±3.0	0.81±0.02***
5	≥ 9001	75	9566.3±75.2	197.6±13.1***	11841.9±36.6	444.6±9.9***	76.7±4.4	0.79±0.02***
Average		391	7918.4±66.3	158.0±5.9***	9078.4±163.4	416.7±4.9***	75.3±1.9	0.89±0.01***

***p<0.001.

The optimal duration of the service period was characterized by cows of the first group - 87.8 days, with a productivity below 6000 kg of milk. As the milk yield increased, the duration of the service period in cows of groups II, in relation to the first, increased by 36.7 days (+41.8%) ($p<0.001$). In highly productive first-calf heifers with a milk yield of more than 9001 kg, the service period was the longest and amounted to 197.6 days (+109.8 days, or 125.1%) ($p<0.001$) and the calving interval to 444.6 days (+90.3 days or +25.5%).

A similar trend was also observed during the calving interval. The calving interval within the biological cycle (354.3 days) was observed in low productivity first-calf heifers with a milk yield of less than 6000 kg of milk. In cows with more than 6000 kg of milk, the duration of the calving interval was more than 400 days, which exceeded the economically justified length of the calving interval (365 days) by 34.5-90.3 days (8.9-25.5%).

According to the regression coefficients, on average, each increase in milk yield by 1000 kg increases the duration of the service period by 27.6 days, the calving interval by 20.9 days, which worsens such an important indicator as "calves yield".

The highest coefficient of reproductive ability was noted in first-calf heifers with milk yield below 6000 kg - 1.03. When the milk yield increased to 8000 kg of milk, the coefficient of reproductive capacity reliably decreased by 0.07-0.16 (7.3-15.5%) ($p<0.01$). When increasing milk yield to 9000 kg of milk, the coefficient of reproductive capacity reliably decreased by 0.22 (21.4%) ($p<0.001$). In first-calf heifers with a milk yield of more than 9000 kg, the reproductive ability coefficient was the lowest - 0.79, which is 0.22 (23.3%) less compared to low-producing animals ($p<0.001$). The least calves were obtained from highly productive cows - 76.7 heads per 100 cows per years, which is less with 21.7 heads (-22.1%) compared to the first group

It is believed that the period from calving a cows to its subsequent fertilization (service period), compared with the interval between calving more accurately reveals the physiological possibilities of the reproductive ability of cows, because "the duration of lactation depends on the service period, the duration of the dry period and interval between calving, the regularity of calving and the calves yield per 100 cows, and as a result, the level of milk production and the efficiency of their use". In this regard, the influence of the duration of the service period on the indicators of milk productivity and the reproductive ability of cows was studied (Table 4). The distribution of cows after finishing the first lactation according of the service period shows that 25.5% of the studied livestock had a biologically justified and economically profitable service period (31-90 days).

Table 4. Productivity indicators depending on the length of the service period ($X \pm S_x$)

Group	Graduation by service period, days	n	Service-period, days	Milk yield/305 days of lactation, kg	Milking days	Milk yield for the full lactation, days	Calving interval, days	CCR
1	< 30	13	27.4±1.95	7178.0±550.10	297.5±16.70	7695.8±761.21	315.2±2.00	1.16±0.01
2	30 - 60	40	46.3±1.41	7278.8±215.23	317.4±16.33	7956.4±408.78	334.4±1.70	1.09±0.01
3	61 - 90	56	74.8±1.07	8033.0±207.20	332.6±9.10	8707.2±315.45	361.8±1.69	1.01±0.004
4	91-120	41	105.9±0.85	7876.3±224.70	344.1±7.01	8702.0±136.04	388.8±1.51	0.94±0.01
5	121-150	60	136.0±0.76	7294.1±92.54	352.35±3.97	7936.6±141.35	418.4±1.19	0.88±0.004
6	150-180	53	164.0±0.78	7769.9±114.71	377.4±4.71	8892.7±192.22	448.4±1.35	0.82±0.004
7	181-210	29	192.9±1.10	8080.5±135.06	406.1±9.61	9819.7±269.49	468.5±3.18	0.78±0.006
8	211-240	20	222.0±1.53	8464.6±283.57	411.9±13.60	10421.4±467.68	504.6±2.11	0.72±0.003
9	241-270	22	253.0±1.74	8593.3±233.45	430.2±17.50	11090.5±676.43	520.3±12.48	0.71±0.02
10	271-300	19	283.6±2.18	8777.5±243.96	489.2±8.14	10358.8±273.3	561.7±2.36	0.65±0.004
11	> 300	27	363.4±6.65	8828.1±165.60	575.8±15.60.	12964.3±515.5	637.8±14.52	0.57±0.012
Average		380	157.6±3.26	7868.3±54.58	364.9±3.53	8795.3±99.95	422.4±2.76	0.89±0.005

In cows of all these groups, with relatively low milk productivity, compared with the average value for the sample, the reproduction rates were high - the coefficient of reproductive capacity from 1.01 to 1.16 (+0.07 ... + 0.15), birth rate - from 96.8 to 112.1 (+6.2%…+15.8%). More than half of the analyzed cows (71.3%) had a service period longer than 91 days, of these, 32.5% were animals with a service period of more than 121 days, showed higher milk productivity - 8258.3 kg of milk for 305 days of lactation and 10212 kg of milk for full lactation (+390.0 kg and +1416.7 kg of milk to the average value for the sample), but low indicators whether in terms of reproducibility. Their reproductive ability coefficient decreased by 0.16 and the yield of calves by 12.5 calves.

Results (Figure 1) regarding the yield of calves state that the increase in the duration of the service period significantly decreases the birth coefficient, with a very high negative correlation persisting (r = -99.9), which confirms that an among the main conditions for accelerating the reproduction rate of the livestock in the household and for increasing the economic efficiency of the branch is that the cows to bee inseminated fertile in the first 2-3 months after calving.

Agalakova & Netecha (2011) related that both a shortened to 30 days and extended service period of more than 90 days negatively affect the productivity and reproductive functions of animals.

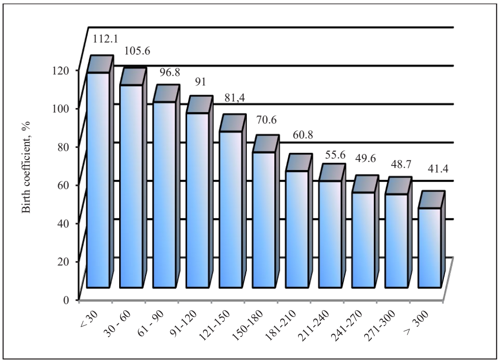

Figure 1. Birth coefficient dynamics depending of the duration of service-period

Table 5 presents the results of the regression analysis between the duration of the service period, milk productivity and the functional activity of the reproductive system in the analyzed cow population.

Table 5. Regression coefficient between service period, milk productivity and the main reproductive indicators of Holstein breed cows' population

Indices		R
Milk productivity for 305 days, kg	Service period, days	97
Milk productivity for full lactation, kg	Service period, days	4.5
Duration of lactation, days	Service period, days	0.53
Calving interval, days	Service period, days	0.75
Coeficient of reproductive capacity	Service period, days	- 0.002
Birth coefficient of, %	Service period, days	- 0.27

Thus, when increasing the length of the service period, milk yield for 305 days of lactation increases by 9.7 kg/day (p<0.01), milk yield for full lactation - by 4.5 kg/day ($p<0.01$), the duration of the lactation increases by 0.53 days/day ($p<0.01$), the calving interval by 0.75 days/day ($p<0.01$). Because of this, the reproduction coefficient decreases by 0.02 ($p<0.001$) and the birth coefficient decreases by 0.27 ($p<0.01$). From these data, it is obvious that increasing the length of the service period and prolonging lactation contribute to increasing the amount of milk per lactation. Similar results were obtained by Popescu (2014). But, according to the obtained data (Table 4), the increase of these two indices (SP, CI) is not profitable, because at the same time it leads to the slowing down of the reproduction rate of the herd, due to the loss of calves, as a whole on the herd.

Dispersion analysis detected the influence of the factor (η^2x - the level of milk productivity) on the duration of the service period was 18.0% ($p<0.001$), on the duration of the calving interval - 15.6% ($p<0.001$), on the reproductive capacity coefficient - 25.6% ($p<0.001$) and on the yield of calves - 35.6% ($p<0.001$).

CONCLUSIONS

With the age of cows, milk increases, but simultaneously, rises the duration of the service-period and the calving interval. The increase of the milk productivity is accompanied by the decrease in the reproductive capacity of cows.

The optimal parameters for the duration of the service period and the calving interval were distinguished by cows that have finished their first lactation, with low milk productivity (yield less than 6000 kg of milk), at which the animals were characterized by the maximum indicators of the birth coefficient and reproductive capacity coefficient.

In cows with a milk yield of at least 9001 kg, a significant increase in the length of the service period (+109.8 days) and the calving interval (+90.3 days) was established, but the reproductive capacity coefficient decreased by 0.22 and the number of calves per 100 cows/year by 21.7 heads.

Increasing of the service period moderately influences milk productivity, but significantly some reproductive indicators of the herd - the duration of the calving interval increases, in the same situation, the coefficient of reproductive capacity and the birth coefficient decreases.

The influence of the level of milk productivity (η^2_x) on the duration of the service period was 18.0% ($p<0.001$), on the duration of the calving interval 15.6% ($p<0.001$), on the reproductive capacity coefficient 25.6% ($p<0.001$), on the birth coefficient 35.6% ($p<0.001$).

We recommend livestock holdings to monitor the duration of the service period, as extending of this period is economically unprofitable for specialized dairy cattle farms.

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EFFECT OF BREED, AGE AND FOOD ON REPRODUCTIVE EFFICIENCY OF FEMALE SHEEP OF THE TSIGAI - RUSTY VARIETY PUREBREED AND THEIR CROSS WITH THE SUFFOLK AND GERMAN BLACKFACE

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Abstract

Fertility, prolificacy and weaning rate were compared for Tsigai - rusty variety (TIRU) purebreed ewes and their Suffolk (50%) × German Blackface (37.5%) x Tsigai - rusty variety (12.5%) (S x BF x TIRU) contemporaries, mated at 8 and 18 months of age. The fecundity, prolificacy and weaning rate for animals mated at 8 months of age and fed in the shelter with hay and corn-barley based concentrate was 78.43%, 105.0% and 78.57% to females from TIRU and 80.0%, 100.0% and 54.17% for animals of the S x BF x TIRU genotype, respectively. For animals of the two breeds mated at 18 months of age and grazing on pastures, the fecundity, prolificacy and weaning rate was 64.71%, 100.0% and 87.88% to females from TIRU and 73.33%, 104.55% and 78.26% for animals of the S x BF x TIRU genotype, respectively. 88.24% females from TIRU and 90.0% from S x BF x TIRU genotype, mated at 8 months, have lambed once up to age of 23-24 months, and 56.86% females from TIRU and 56.66% from the animals from S x BF x TIRU genotype have lambed twice.

Key words: *early bred, efficiency, genotype, reproduction, Tsigai - rusty variety.*

INTRODUCTION

The Tsigai breed is a sheep breed raised for milk, meat and wool production, the breeding area being the regions of Central, Eastern and Southern Europe (Cinkulov et al., 2008).
In Romania, the Tsigai sheep is the second most important breed, the main productions for which it is raised are milk and meat, as the interest in wool production has lost its importance due to the low price obtained for wool sales (Ilișiu et al., 2013).
Due to the increasing of the lack of labor in the sheep breeding sector in recent years, many sheep breeders are focusing on improving meat production. The methods used to increase meat production within a race are multiple, but feeding technologies on the one hand and improvement of reproductive performances of sheep on the other hand, are key factors that can contribute to improving of meat production.
As stated by Gavojdian et al. (2015), fertility related traits and lambs weaning rates represent the main indicators for profitability, when meat sheep are concerned.
From the point of view of the sheep breeders, the profitability of farms can be improved by introducing young females at the age of 12-14 months to reproduction. Mating young females of sheep to lamb first time at 17-19 months compared to sheep lambing at 24 months for the first time, can contribute to improving flock profitability.
By introducing the young female to reproduction a few months earlier than usual, the sheep breeders can manage better the sheep flock, find out earlier information about the reproductive traits of the females, being able to eliminate infertile females from reproduction.
As stated by Hutchison et al. (2022), the differences of reproductive rate between young female and mature ewes are by 50%. The reproductive performances of young females can be influenced by genotype. Fogarty et al. (2007) suggest that age and body weight at first mating are attribute which influences ewe lamb reproductive performance.

Within a breed or genotype, the body weight is probably the most important factor on reproductive performances of young sheep. Some authors mentions the existence of positive correlations between the body weight of young sheep at first mating and their reproductive characteristics (Kenyon et al., 2014; Paganoni et al., 2014; Edwards et al., 2017; Nieto et al., 2019; Shorten et al., 2021; Thompson et al., 2021; Haslin et al., 2022).

The aim of current study is to determine the effect of breed, age and food on reproductive performances of Tsigai breed - rusty variety and their cross with Suffolk and German Blackface.

MATERIALS AND METHODS

Animals and location

The two experiments described here were carried out using ewe lambs 8 months of age and 18 months-old ewes of the Tsigai - rusty variety purebred (TIRU) and their Suffolk (50%) x German Blackface (37.5%) x Tsigai - rusty variety (12.5%) (S x BF x TIRU) contemporaries. The experiments were conducted in the Experimental Base Reghin of Research Institute for Sheep and Goat Palas Constanta, Mures County, 46°46' N/ 22°42'E; 395 m altitude; annual rain fall varies between 650-700 mm; average temperatures 19/–3°C during summer/winter). Reproductive performances during early breeding and at 18 months of age were observed over 2 years on female sheep born January-March 2020. A total of 51 ewe lambs of TIRU breed and 30 ewe lambs of S x BF x TIRU genotype were included. Up to 16 months, all sheep were raised in the shelter. After this time, the sheep grazed on pastures until beginning of December. Starting on the 1st October in the first year (8 to 9 months age) and 1st of August of the 2nd year (18-19 months age) and for a mating period of 45 days, females of the two breeds, were mated with rams of the TIRU sheep breed and S x BF x TIRU genotype, respectively. In the 1st year, after mating, all females were fed with 1.5 kg hay and 0.50 kg corn-barley concentrate (50%-50%) (14% crude protein content) per head per day during first 3 months of pregnancy and with 2.0 kg hay and 0.8 kg corn-barley concentrate (50%-50%) in late pregnancy and early lactation. The age in days, live weight at mating and at lambing, the incidence of lambing and the number of lambs born were recorded.

For mating at 18 months of age, the two groups of animals used in the early breeding experiment were available. In total, fifty-one and thirty sheep of respectively TIRU breed and the S x BF x TIRU genotype were used and data on both groups and both mating ages are presented here. Starting on May 15th and until August 15th, animals of the two breeds were grazed on pasture in the same flock. In mid-August, the animals were separated in two flocks and were grazed on pastures, and were organized controlled mating with rams from TIRU breed and S x BF x TIRU genotype, respectively. In mating period (45 days), the food was supplemented for each flock with 0.3 kg corn-barley-based concentrate (14% crude protein content)/head/day. In late pregnancy (December-Januar) all sheep were fed per head per day with 1.5 kg hay and 0.5 kg corn-barley-based concentrate and in early lactation with 2.0 kg hay and 0.8 kg corn-barley-based concentrate. Live weight at mating, the incidence of lambing and the number of lambs born were recorded.

In order to determine the effect of breed, age and food on reproductive efficiency of female sheep of the Tsigai - rusty variety purebreed and their cross with the Suffolk and German Blackface, the mean comparisons between the two groups of the variables were carried out using independent samples Student t-test of the JASP procedure.

RESULTS AND DISCUSSIONS

This experiment investigated the effect of breed, age and food on breeding performances of young females mated at 8 months of age and mature ewes (mated at 18 months age), and performances of their progeny.

Table 1 shows overall values and differences between the two breeds structures (TIRU and S x BF x TIRU) in body weight at mating, age at mating, the number of sheep lambing and mean litter size of young females mated at an early age.

Table 1. Reproduction indices for females mated at 8 months of ages

Specification	TIRU	S x BF x TIRU	Overall
Number of sheep joined (head)	51	30	81
Body weight of sheep at mating (kg)	40.62 ± 0.61^{A}	52.65 ± 1.21^{B}	45.08 ± 0.87
Age (days)	234.92 ± 1.28	240.60 ± 1.65	237.03 ± 1.05
Body weight of sheep at birth (kg)	51.77 ± 0.76^{A}	64.73 ± 1.18^{B}	56.57 ± 0.95
Number of sheep lambing (head)	40.0	24.0	64.0
Number of lambs obtained (head)	42.0	24.0	66.0
Number of lambs weaned (head)	33.0	13.0	46.0
Fecundity (%)	78.43	80.0	79.01
Prolificacy (%)	105.0	100.0	103.13
Weaning rate (%)	78.57^{a}	54.17^{b}	69.70

$^{a, b}$ - Means in the same line with different superscripts are significantly different (p<0.5);
$^{A, B}$ - Means in the same line with different superscripts are significantly different (p<0.001).

With regard at live weight at mating, between the two groups of young females mated at 8 months were recorded significant differences (p<0.001) in favour of S x BF x TIRU. Ewe lambs from Tsigai breed had at 8 months an average body weight lower by 29.61% than their contemporaries S x BF x TIRU, but the fecundity was not affected by this difference in body weight (p>0.05).

Significant difference (p<0.5) is observed on the weaning rate of lambs from the two genotype, which is higher with 26.87% to Tsigai breed.

The average live weight of the whole flock at breeding was 45.08 kg at 8 months (Table 1) and 43.40 kg at 18 months (Table 2).

Some authors (Moore et al., 1978; Meyer & French, 1979; Craig, 1982; McMillan & Moore, 1983), have shown, that increasing body weight at the beginning of the mating period has led to the increase of mating activity.

Concerning reproductive performances of ewes mating at 18 months of age, data on the reproduction indices are shown in Table 2. For the 2nd mating, the average body weight at breeding for ewes lambs from Tsigai breed was close to the one at 8 months (-0.34 kg), and that of the S x BF x TIRU was lower than the one at 8 months with 7.48% (-3.94 kg). It should be mentioned that before the 1st lambing and until the lambs were weaned, the sheep were kept in the stable and fed with concentrates and hill hay, and after, until the beginning of the 2nd mating, the sheep grazed on pasture of medium quality, whose floristic composition is constituted by 90% of grasses from the spontaneous flora (in which the proportion of *Deschampsia cespitosa* is over 50%, together with *Festuca pratensis*, *Dactilylis glomerata* and *Lolium perenne*) and 10% legumes (*Trifolium repens*).

Table 2. Reproduction indices for females mated at 18 months of ages

Specification	TIRU	S x BF x TIRU	Overall
Number of sheep joined (head)	51	30	81
Body weight at mating (kg)	40.28 ± 0.73^{A}	48.71 ± 1.17^{B}	43.40 ± 0.78
Age (days)	528.92 ± 1.28	534.60 ± 1.65	531.03 ± 1.05
Number of sheep lambing (head)	33.0	22.0	55.0
Number of lambs obtained (head)	33.0	23.0	56.0
Number of lambs weaned (head)	29.0	18.0	47.0
Fecundity (%)	64.71	73.33	67.90
Prolificacy (%)	100.00	104.55	101.82
Weaning rate (%)	87.88	78.26	83.93

$^{A, B}$ - Means in the same line with different superscripts are significantly different (p<0.001).

The fecundity of ewes at 18 months was lower than at 8 months for both breed structures. For Tsigai breed, although the average body weight at 18 months was lower with 0.83% than to the one at 8 months, the fertility was lower with 13.72%. The conception rates for the Tsigai mature ewes were lower than those, obtained by Gavojdian et al. (2015), for the breed. On the other hand, the average body weight at 18 months was lower with 7.48% to S x BF x TIRU compared to 8 months, and the fertility rate was lower with 6.7%.

The average fecundity rate for the whole flock was lower at 18 months with 11.11% than at 8 months, while the weaning rate of the whole flock at 18 months was 83.93%, higher with 20.29% than at 8 months.

This lower fertility of 64.71% for the group of Tsigai breed and 73.33% for the group of females S x BF x TIRU, for sheep mated at 18 months of age, (lambed for first time at 13-14 months of age and suckled their lambs for at least a two months following early breeding) is with apparently detrimental effects.

When compared the two breed structures, the weaning rate were lower at 8 and 18 months for ewe lambs and mature ewes from S x BF x TIRU (54.17% and 78.26%, respectively) compared to their contemporaries from Tsigai breed (78.4% and 87.88%, respectively). On the other hand, when compared the weaning rate between the two age categories (8 and 18 months), it is observed that the effects of age at breeding on weaning rate is significant ($p<0.05$) to adult ewes compared to ewe lambs. This is probably due an improved milk production of ewes at 18 months, but we consider that here are more studies needed to highlight the effect of age at first mating on weaning rates of lambs.

Table 3. Reproduction indices for females mated at 8 and 18 months of ages

Specification	8 months age	18 months age
Number of sheep joined (head)	81	81
Body weight at mating (kg)	45.08 ± 0.87^a	43.40 ± 0.78^b
Age (days)	237.03 ± 1.05	531.03 ± 1.05
Number of sheep lambing (head)	64.0	55.0
Number of lambs obtained (head)	66.0	56.0
Number of lambs weaned (head)	46.0	47.0
Fecundity (%)	79.01	67.90
Prolificacy (%)	103.13	101.82
Weaning rate (%)	69.70^a	83.93^b

$^{a, b}$ - Means in the same line with different superscripts are significantly different ($p<0.5$).

The Table 4 highlights the fact that, up to the age of 24 months, 88.24% of the sheep from the Tsigai breed and 90% of the S x BF x TIRU crossbred sheep lambed at least once, while the share for the sheep lambed twice is almost identical for the two breed structures (56.86% at Tsigai breed *vs*. 56.66% at S x BF x TIRU).

Table 4. The number of sheep that lambed up to 24 months age

Specification	TIRU	S x BF x TIRU	Overall
Number of sheep joined (head)	51	30	81
Lambed once (no.)	45.0	27.0	72.0
(%)	(88.24)	(90.0)	(88.89)
Lambed twice (no.)	29.0	17.0	46.0
(%)	(56.86)	(56.66)	(46.79)

In mature ewes, the body weight of the ewe at the time of mating is lower at 18 months than to 8 months of age, and negatively associated with the lamb birth weight, lamb weight at weaning and average daily gain from birth to weaning (Table 5). In the present experiment, the body weight of females at mating influence lamb birth weight, lamb weight at weaning and average daily gain. The obtained results of the reproductive performances of young females compared to mature ewes highlight the fact that the performance of young ewes can contribute to improving the profitability of sheep farming.

Table 5. The body weight evolution of lambs from birth up to weaning in the years 2021-2022

Specification	N	TIRU	N	S x BF x TIRU
Year 2021(lambs from 8 months ewes lambs)				
Weight at birth (kg)	33	3.78 ± 0.10^A	13	4.48 ± 0.15^B
Weight at weaning (kg)	33	18.32 ± 0.63^c	13	22.29 ± 1.67^d
ADG g/day	33	196.43 ± 7.64	13	219.40 ± 19.12
Age at weaning (days)	33	73.97 ± 1.42^a	13	80.62 ± 2.54^b
Year 2022 (lambs from 18 months ewes)				
Weight at birth (kg)	29	3.70 ± 0.01^c	18	4.34 ± 0.21^d
Weight at weaning (kg)	29	14.03 ± 0.38^A	18	17.25 ± 0.60^B
ADG g/day	29	139.48 ± 6.94^A	18	213.27 ± 15.74^B
Age at weaning (days)	29	76.86 ± 2.72	18	78.67 ± 19.78

$^{a, b}$ - Means in the same line with different superscripts are significantly different ($p<0.5$);
$^{c, d}$ - Means in the same line with different superscripts are significantly different ($p<0.01$);
$^{A, B}$ - Means in the same line with different superscripts are significantly different ($p<0.001$).

CONCLUSIONS

The present study highlighted that mating young females of sheep at early age can contribute to improving ewe performance throughout the productive life. The genotype, age, body weight of the young females at first mating and nutrition during gestation period can significantly influence ewe and lamb performance to weaning, as well as ewe reproductive performances. Under Romanian condition, there are a little amount of researches on the productive and reproductive performances of females introduced early for reproduction, therefore further research are needed to help the improvement of farm management.

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MORPHOLOGICAL CHANGES OF THE REPRODUCTIVE ORGANS IN DOMESTIC CHICKEN SUFFERED FROM INFECTIOUS BRONCHITIS, BASED ON AN EXCESS OF VITAMIN D3 IN THE DIET

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Abstract

In private mini-poultry farms, poultry that have recovered from infectious bronchitis are often not culled. Changes in the organs of the reproductive system of adult chickens and roosters that recovered after an outbreak of infectious bronchitis were studied. It was found that in most of the flock, egg productivity and condition quality of eggs were preserved in laying hens (69%), and in roosters - fertilization ability (after hatching the eggs, hens hatched condition chicks with 80% hatchability). In 31% of hens, egg productivity and/or egg quality was impaired: reduced laying; hens laid eggs with soft and/or deformed shells; there was no clear boundary between the dense and liquid layers of the egg white; the share of second-grade eggs has increased. During the post-slaughter examination of chicken carcasses, various pathologies of the reproductive organs were found: retention of the egg in the oviduct and damage to its walls (it is possible that this is due to calcareous growths on the eggshell); ovarian cysts; atresia of mature follicles.

Key words: *domestic chickens, egg production, infectious bronchitis, ovariitis, reproductive system, salpingitis.*

INTRODUCTION

Scientists from all over the world are investigating various aspects of infectious bronchitis in chickens (Hoerr, 2021; Shao et al., 2020; Bande et al., 2017; Khataby et al., 2016; Chacón et al., 2014; Cook et al., 2012; Jackwood, 2012; Chousalkar et al., 2007; Raj & Jones, 1997). According to Amarasinghe et al. (2017), Han et al. (2017) and Cavanagh, (2007), the coronavirus, which is the causative agent of the disease, damages the macrophage structures of the poultry body.
Researchers Shahnas et al. (2020) define infectious bronchitis of chickens as a polysystemic disease with suppression of immunity. In case of spontaneous infection of poultry with its causative agent, part of the flock is resistant, which is determined by the status of the immune system (Smith et al., 2015). The instructions for fight against infectious bronchitis in commercial poultry farm, provide for the depopulation of poultry that were infected (Berezovskyi, 2017). The credo of private mini-poultry farms, as a rule, is the search for schemes for poultry saveting (rehabilitation). This approach is justified by the fact that after recovery from infectious bronchitis, the bird have immunity to a specific strain of the causative agent of the disease for a period of at least 5 months (Stehnii et al., 2013). But in such a bird, there are various consequences (long-term and temporary), which indicate that the birds were sick of infectious bronchitis in the past. Among them are changes in the reproductive system of chickens, including impaired or lost egg productivity (Liakhovych et al., 2022; Prokudina, 2015). Dynamic monitoring of birds with infectious bronchitis in the conditions of a specific poultry farm contributes to timely detection and objective assessment of its consequences. Characterization of morphological changes of chickens reproductive organs during infectious bronchitis is an important informative component of understanding the patterns of reproduction processes in reconvalescended birds, the basis for solving practical problems of flock reproduction, assessment and prognosing of egg productivity of flocks.

MATERIALS AND METHODS

We made research by two stages. At the first stage, the level of egg productivity and egg quality was determined in adult crossbred hens reconvalescended from viral bronchitis due to spontaneous infection (aged from 5 months to 2.5 years). Poultry was kept in a specially equipped place in compliance with standard animal hygiene and veterinary sanitary parameters. The chickens were fed mixtures of cereal grain and/or its waste (shredded wheat, barley, corn); chopped fodder beets, carrots, potatoes; in summer - with green mass of milkweed, cabbage leaves, chopped pumpkins and zucchini. Periodically, the feeding ration was supplemented with a cake of sunflower seeds, flax, fish meal, dairy waste; in order to correct calcium and phosphorus metabolism, which are involved, in particular, in the formation of a strong egg shell, they were fed synthetic preparation of vitamin D. Chickens were supplied with water without restriction. A walking platform was equipped around the perimeter of the aviary. At the second stage, during the post-mortem examination of the slaughtered chickens carcasses (n = 17) and roosters (n = 3), which was performed in accordance with existing requirements (Yatsenko et al., 2015), the organs of reproductive system were examined in detail. Their anatomical preparation was carried out under the control of low magnification optical lenses. The research was conducted according to the scientific topics of the Department of Normal and Pathological Morphology of the State Biotechnology University. Methods of clinical observation, autopsy, anatomical preparation and analysis were used.

RESULTS AND DISCUSSIONS

Evaluation of egg productivity and egg quality of the researched laying hens showed a decrease in the amount of conditioned eggs and, accordingly, an increase in non-conditioned and low-grade eggs (nutritional inferiority of eggs with shell defects; II category eggs weighing at least 44 g; eggs weighing not less than 43 g, which are sold in Ukraine under the name "small" (Tiutiun, & Pototskyi, 2011) (Figure 1). According to Quinteros et al. (2022), a quarter of chickens infected with the infectious bronchitis virus laid eggs with a soft shell, and 10% - with a rough surface.

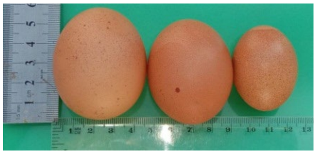

Figure 1. The appearance of substandard eggs from chickens that were reconvalescended from infectious bronchitis

With the appearance of small eggs, hens were suspected of egg-laying abnormalities, in particular, due to stenosis of the oviduct. A narrowing of the oviduct lumen in its cranial part, caused by post-necrotic scarring of the wall, was diagnosed during the post-mortem examination of the culled chickens. There were violation of the formation of the eggshell, including, with the absence of its calcification in 20% of the studied chickens (Figure 2). Such eggs were easily broken immediately in the nest, leaving corresponding traces of fragments of the inner and outer layers of the shell and the moist sticky mass of protein and yolk. To obtain native preparations of these eggs, the corresponding female chickens were isolated in separate sectors of the poultry house, provided with a nest with soft porous filling, in which the egg retained its integrity after laying.

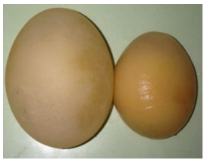

Figure 2. Appearance of eggs with non-calcified shells from chickens after infectious bronchitis. Native sample

Chickens ate the remains of uncalcified eggs. As it known, chicken eggs contain various hormones: estrogen, progesterone, testosterone (Aslam et al., 2013). Chicken egg is rich in proteins, vitamins, minerals, fatty acids (Milinsk et al., 2003; Qi & Sim, 1998). An excess of these substances, in particular, steroid hormones and vitamin E, is harmful to the reproductive system of the bird. It was established that in some chickens there was an overdose of synthetic vitamin D. This was evidenced, in particular, by the thickening of the eggshell (it reached 0.43 mm in some parts of it) and hilly chalk growths on it. Such an eggshell injured the mucous membrane of the oviduct of chickens, disturbed its peristalsis, which caused a delay in laying the egg. Complicated egg laying took place. Calcified outgrowths interfered with the creation of the effect of sliding on the mucous membrane, so egg-laying caused pain in female hens. In some culled chickens, post-mortem examination showed signs of delayed egg laying due to hypercalcified and deformed shell (Figure 3).

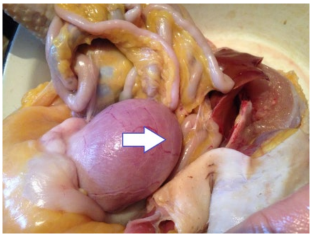

Figure 3. Visceral organs of domestic chicken. Autopsy. Retention of the egg in the uterus (arrow)

The uterus stretched and lowered, its wall became thinner and lost tone (due to the delay of the egg in fallopian tube (blood stasis developed in its venous vessels) (Figure 4).
The egg retained its integrity after laying.

Figure 4. A fragment of the oviduct and uterus with a retained egg in a domestic hen reconvalescended from infectious bronchitis. Native sample

Due to the slow movement of the egg in the fallopian tube, the secretion of the glandular structures of the mucous membrane was disturbed. The delay of the egg in the uterus of the fallopian tube had a particularly negative effect on the neurovascular layer of its muscular membrane. It is located between the inner (circular) and outer (longitudinal) layers and is a wide layer of fibrous connective tissue (Zhyhalova et al., 2008; Bakst, 1998). These violations led to: atony and stretching of the wall and other parts of the fallopian tube due to compressive atrophy of the muscular elements; the mesentery of the fallopian tube was also stretched (Figure 5).

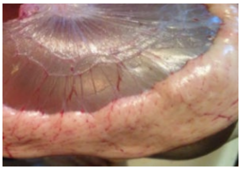

Figure 5. A fragment of an eviscerated fallopian tube with a mesentery of an adult domestic chicken reconvalescended from infectious bronchitis (venous stasis of vessels of the serous membrane and mesentery; stretching of the wall). Native sample

We finded changes classified as vascular congestion (post-compressive internal) and muco-catarrhal ovariitis during macroscopic examination of the oviduct mucous membrane in slaughtered hens with a history of complicated egg-laying due to large egg size and/or deformed hypercalcified shell (Figure 6).

Figure 6. Viev of the mucous membrane surface of the oviduct fragment of the domestic hen with signs of local internal post-compressive congestion and mucocatarrhal ovariitis (arrow) due to egg retention. Native sample

It is known that in birds, the uterus of the oviduct narrows at the border with its vagina and forms a uterine-vaginal junction, which plays an important role in sperm deposition (Wahabu, 2016; Kot, 2011; Bondarenko, 1998; Bakst, 1998). Delay of the egg in the fallopian tube due to stretching and loss of tone of its wall leads to a violation of this depositing function (as a result - loss of fertility in females). Normally, in chickens, only one (the largest) follicle per day is ready for ovulation: preovulatory follicles ovulate in order of size due to a surge of luteinizing hormone (Cunningham et al., 1984). Signs of edema developed in the vascular membrane of preovulatory follicles according to delay of the formed egg in the egg-bearing tract of chickens (Figure 7).

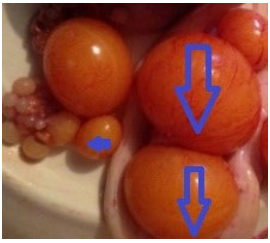

Figure 7. Preovulatory follicles (down arrow): blood stagnation in the vascular membrane; small yellow follicles during egg retention in chickens (left arrow)

In individual chickens, double oviposition per day took place (double oviposition on one day) with the laying of eggs of various sizes, including small and/or without calcified shells. Three one-and-a-half-year-old hens with stopped egg-laying had increased abdominal volume and difficulty breathing. They were culled, and during the post-mortem examination, changes typical for yolk peritonitis were diagnosed, with the presence of sticky, thin, dirty-yellow contents in the abdominal cavity (due to the rupture of yolk follicles). Defective, compacted, stone-like yolk follicles in the capsule, which were easily ruptured, were also found (Figure 8).

Figure 8. The content of the encapsulated yolk follicle in an adult chicken suffering from infectious bronchitis

Adhesion ovarioperitonitis was diagnosed postmortem in one slaughtered chicken; the other two have salpingoperitonitis with adhesive obliteration of the peritoneum and the serous lining of the intestinal tract. In these chickens, patho-anatomical signs of non-specific intoxication of the body of varying severity (general cyanosis, toxic liver dystrophy, ascites, pericarditis, myocardial dystrophy, granular renal dystrophy) were detected. A post-mortem examination revealed a monocystic lesion of the oviduct in one 5.5-month-old immature hen, which had no clinical manifestations characteristic of infectious bronchitis (Figure 9). At the same time, hyperplasia of the bursa of Fabricius was observed, which indicates a sufficient immune status in this individual bird.

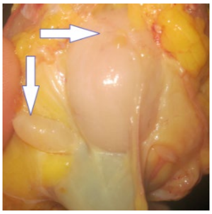

Figure 9. An oviduct cyst (down arrow) and a bursa of Fabricius (right arrow) in a 5.5-month-old hen which has not yet laid eggs

Polycystic ovary was diagnosed in three culled hens with stopped egg-laying during the post-mortem examination (Figure 10). Multiple follicular cysts of different sizes surrounded by a compacted capsule of connective tissue elements were found. Their lumen contained a liquid or oily mass. Torsions of individual cysts with rupture of the capsule were observed.

Figure 10. Polycystic ovary an adult chicen suffering from infectious bronchitis

A follicular ovarian cyst indicates a violation of the normal ovulation process: when a mature follicle does not ovulate due to the death of an egg in it. Disintegration of follicles at different stages of their development was detected. This pathology was classified as follicular atresia. Similar changes are described by researchers Hassan et al. (2021), Pereira et al. (2019), Boroomand et al. (2012), in particular, in the experimental infection of chickens with a highly pathogenic strain of infectious bronchitis virus. Scientists Mueller et al. (2022), Zhong et al. (2016) indicate the pathogenicity of the virulent infectious bronchitis virus isolate YN on the ovary and oviduct of chickens. Zhang et al. (2020) observed changes in the reproductive system and egg production of chickens infected with infectious bronchitis virus TW type I. A peculiar finding was the changes in oviduct mucous membrane during the preparation of the oviduct of a 2.5-year-old hen, culled due to cessation of egg-laying (Figure 11). Necrotic salpingitis was diagnosed.

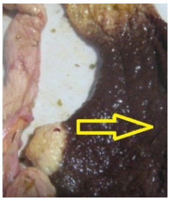

Figure 11. A fragment of the dissected oviduct (from the mucous membrane side) of an adult hen with stopped egg-laying after remissions of infectious bronchitis: necrotic salpingitis (arrow)

This individual had classic respiratory signs of infectious bronchitis during his lifetime. After the first two remissions of the disease, the hen had a temporary cessation, and later - a resumption of egg laying. There were hyperpigmented small spots of dark brown-red color on the shell of the laid eggs.
Under the dynamic monitoring of productivity indicators and the morphological state of the reproductive organs of the studied poultry, these indicators were evaluated in roosters. After the slaughter of a 7-month-old rooster, an examination of the organs of the thoraco-abdominal cavity revealed that the testicles are anatomically correct (at the level of the last three ribs), and have an ovoid shape; the right testicle is smaller than the left; the color of the surface of the testes is pale yellowish (Figure 12), the consistency is elastic.

Figure 12. View of the testicles of a 6-month-old, immature rooster reconvalescended from infectious bronchitis

The indicated parameters of the macroscopic characteristics of the testicles of the investigated rooster coincide with the data of Althnaian (2022), Mfoundou et al. (2022), Razi et al. (2010). According to Mfoundou et al. (2022), in immature roosters the testes are yellowish or pink in color and the left testis is always larger than the right.
Fertilizing ability was preserved in the two studied adult roosters: under natural conditions of incubation of eggs by hens, 80% hatching of chicks with high viability took place. A postmortem examination of a one-and-a-half-year-old rooster revealed that the testicles were anatomically placed correctly, had a bean-like shape, and a slightly increased volume (this indicator was determined by the following test, which is used in the macroscopic diagnosis of the parameters of bean-shaped organs: the testicle was dissected from its convex part to 2/3 of the depth of the organ, after which the dissected halves were compared - they deviated somewhat from the cut line, which indicates an increase in the organ).
This increase in testes is a morphological criterion, in particular, of spermatogenic activity. Dyscirculatory disorders were observed in the vascular membrane of the testicular capsule: hyperemia, hemostasis with the effect of small hemorrhages (Figure 13), which developed under general cyanosis in the background of keeping this male rooster in hypodynamic conditions.

Figure 13. View of the testicles of a one-and-half-year-old rooster: hemodynamic disorders of the choroid

CONCLUSIONS

The study of the correlation of egg productivity and the morphological state of the reproductive organs of chickens helps to objectively assess the influence of various factors and their relationship on the bird's body. With an excess of vitamin D in the diet of the researched reconvalescended from infectious bronchitis egg-laying hens, hypercalcification and deformation of the eggshell were observed. This, in combination with morphological changes in the oviduct of chickens caused by the influence of the infectious bronchitis virus (stenosis on the background of scarring), provoked complicated egg laying. Analysis of the effects of factors affecting the reduction, disruption or cessation of egg-laying in hens reconvalescunted from infectious bronchitis contribute to the optimization of the production process, prognosing the reproductive and productive qualities of the flock. Also, this information supplements the data on the differential diagnosis of pathologies of the reproductive organs of poultry.

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PHYSICAL-CHEMICAL PARAMETERS OF CARPATHIAN GOAT COLOSTRUM AND MILK

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Abstract

Metabolism, a manifestation specific to life, represents the set of physical and chemical processes through which living matter achieves continuous renewal to function and organize the specific activity. The physical-chemical composition of goat milk is conditioned by breed, individuality, area, and age. This study analyzes the physical-chemical composition of milk from a population of Carpathian goat breed, in the first seven postpartum days (first, third, and seventh day) according to age (primiparous and multiparous) to observe the physical-chemical changes from colostrum to milk. The physical-chemical parameters analyzed were: fat (g/100 g); protein (g/100 g); casein (g/100 g); lactose (g/100 g); solid non-fat - SNF (g/100 g); total dry matter (g/100 g); pH; freezing point (FP); urea mg/dl; somatic cells count - SCC/ml x 1000; total bacteria count - TBC/ml x 1000. Results highlight the intense changes that occur at the physico-chemical level of goat's milk to cover the nutritional requirements of the newborn goat and indicate the health status of the mammary gland.

Key words*: casein, colostrum, fat, metabolism, protein.*

INTRODUCTION

The study of colostrum composition and gradual transition to normal milk is of considerable interest from several points of view. Colostrum indicates the rate at which the epithelial cells gradually take over the function of milk synthesis and also the immune bodies are transferred from the mother to the fetus in association with the globulin fraction of the colostrum, which gives additional importance to the separate analysis of this protein because it passes unchanged from the bloodstream into milk (Woodman, 1921). The first studies performed on the composition of goat colostrum and the transition to normal milk analyzed constituents such as total solid, fat, casein, albumin, globulin, lactose, water, and ash (Bergman & Turner, 1937). Milk protein is one of the most important nutritional components of goat milk (Zhu et al., 2018). In the first five days postpartum, goat milk was considered colostrum because there was a significant difference in the composition of goat milk. After 5 days, it was switched to normal milk when all measured parameters were within normal ranges (Mahmut et al., 2007; Di Chen et al., 2018). The composition of goat colostrum and its evolution to normal milk is essential for the transfer of passive immunity to goat kids and for the development of an optimal formula of milk powder to ensure the nutrient requirements for new-born goat kids in the case of artificial feeding (Constant et al., 1994; Arguello et al., 2004a; Clark et al., 2017). The advantages of artificial feeding are due to the lack of weaning stress which stagnates the growth of the goat kids for 7-8 days. The chemical composition of milk is conditioned by the breed, individuality of the animal, area, age, level, nature of feeding, lactation stage, season, milking duration, and health status (Morand-Fehr et al., 2007). Breed influences goat milk production, when milk or colostrum production is high, the total protein content decreases (Pritchett et al., 1991; Quigley et al., 1994). Evaluation of milk composition showed that two- and three-year-old goats do not have any significant difference in terms of fat, SNF, protein, and lactose. As for four-year-old goats, there are distinct differences in composition where the fat content is extremely high (Ibrahim & Jail, 2022). Somatic cells have no active role in the composition of goat milk and indicate the quality and health of the mammary gland. The general somatic theory tells us that the number of cells increases with the development of lactation (Poutrel & Lerondelle, 1983). The level of somatic cells is significant for goat milk during the lactation period (Haddadi et al., 2006), and physiological status is insignificant for microbially goat milk quality (Saha, 2022).

MATERIALS AND METHODS

This study aimed to identify the physical-chemical parameters of goat milk in a Carpathian goat population depending on the physiological state, during the first seven days postpartum. The Carpathian goat population under study is located in the area of the Trascău Mountains, Alba County. The study included 10 Carpathian goats at the first lactation (primiparous) and 10 Carpathian goats at the second lactation (multiparous). Milk samples were collected from each specimen belonging to the experimental group at 3 different postpartum periods: first day postpartum, third day postpartum, and seventh day postpartum following the model of Chen, D., who takes the composition of goat milk from a Laoshan goat population throughout the lactation period, with an emphasis on analysis of the first seven days postpartum (Chen et al., 2018; Argüello et al., 2006). The udder was pre-washed with warm water and blotted with a disposable paper towel; the goat milk was milked by hand and it was considered that the milking was complete and hygienic for the accuracy of the analyzed data. The milk sample was homogenized and put in sterile containers (50 ml), to be analyzed the physical-chemical parameters of goat milk: fat (g/100 g); protein (g/100 g); casein (g/100 g); lactose (g/100 g); solid non-fat - SNF (g/100 g); total dry matter (g/100 g); pH; freezing point (FP); urea mg/dL; somatic cells count - SCC/mL x 1000; total bacteria count - TBC/mL x 1000. The milk sample was analyzed with the help of the MilkoScan FT (Foss Electric) which is based on Fourier transform infrared technology and provides a wide range of compositional parameters for milk devices (Sánchez, 2007; Saha et al., 2022). Milkoscan is calibrated according to reference methods: ISO 8968-2/IDF 20-2 for protein (ISO, 2014); ISO 1211/IDF for fat (ISO, 2010); ISO 26462/IDF for lactose (ISO, 2010); ISO 6731/IDF for milk solids (ISO, 1989); ISO 108:2002 for freezing point (IDF, 2002); ISO 14637:2004 for urea and pH (ISO, 2004b). The total bacterial count was determined by flow cytometry method using a BactoScan^{TM} according to ISO 21187:2004 (ISO, 2004) and somatic cells count was determined by Fossomatic FC according to ISO 13366-1:2008 (ISO, 2008). ANOVA one-way was used to determine if there were statistically significant differences between means of groups (intragroup and intergroup), followed by Tukey post-hoc test for multiple comparisons (Chende et al., 2022; Matei-Lațiu et al., 2023). Before performing ANOVA one-way, the normal distribution of data was analyzed. Where data did not follow a normal distribution, the Kruskal-Wallis test was used, seconded by the Dunn test. The threshold for statistical significance was set to α=0.05. Data processing was performed in Microsoft Excel for Windows, MS Excel 2016, version 16.0.4266.1001, and GraphPad Prism 8.0.1.

RESULTS AND DISCUSSIONS

Changes in fat content were observed in both primiparous (P) and multiparous (M) groups only when comparing day 1 to day 7 (P-Day 1 vs. P-day 7: P=0.0573 and M-Day 1 vs. M-day 7: P=0.751). Fat content from day 3 and day 7 did not show statistically significant differences (P<0.0001 in both cases). Intergroup comparison (P-Day 1 vs. M-Day 1, P-Day 3 vs. M-Day 3, P-Day 7 vs. M-Day 7,) did not show statistically significant differences (P>0.9999) (p and Figure 1). Fat content is higher in the first days postpartum and remained high until day 5 and reached normal milk goat fat percentage at day 15 (Sánchez-Macías et al., 2014). Compared to mature goat's milk, colostrum has significantly higher contents of protein, fat, minerals, dry matter, and a lower concentration of lactose (Yufang et al., 2021). The stage of lactation had significant effects on the contents of fat, protein, lactose (Kondyli et al., 2007). In general, an increase was observed in protein, fat, ash, SNF, and viscosity, except for lactose and pH, which recorded high values during the first lactation, decreasing until the fourth lactation (Antunac, 2001).

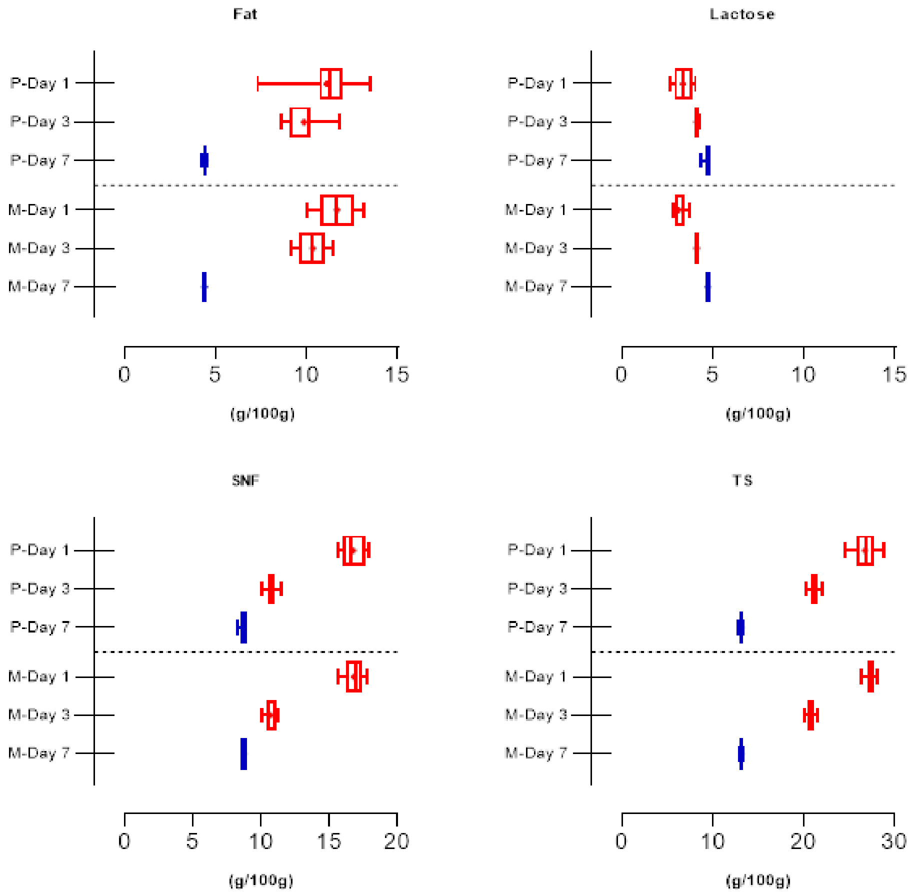

Figure 1. Variations and descriptive statistics of fat, lactose, SNF and TS content determined for primiparous and multiparous group. Red box and whisker plots represent colostrum analysis (day 1 and day 3). Blue box and whisker plots represent milk analysis (day 7). P represents the primiparous group and M represents the multiparous group

Lactose and SNF content registered identical trends to fat content, intragroup analysis showing statistically significant differences only when comparing day 1 to day 7 ($P<0.0001$ both for primiparous and multiparous). Intergroup analysis din dot showed statistically significant differences ($P>0.9999$) (Figure 1).

Ontsouka et al. (2003) observed that lactose production values were lower in colostrum than in mature milk. According to Oltner (1985), we can state that the casein content and urea content in Carpathian goat milk is influenced by the stage of lactation. Lactose content can also be used as an indicator of mammary gland health (Lindmark-Månsson et al., 2006).

The total solids content (TS) presented statistically significant differences in both intragroup analysis (primiparous and multiparous groups) (P-Day 1 vs. P-Day 3; P-Day 1 vs. P-Day 7; P-Day 3 vs. P-Day 7; M-Day 1 vs. M-Day 3; M-Day 1 vs. M-Day 7 and M-Day 3 vs. M-Day 7: $P<0.0001$).

Intergroup analysis (P vs. M) did not show statistically significant differences (P-Day 1 vs. M-Day 1: $P=0.446$; P-Day 3 vs. M-Day 3: $P=0.7307$; P-Day 1 vs. M-Day 1: $P>0.9999$) (Figure 1). SNF content varies slightly during the lactation stage and the change in SNF content is less pronounced, especially in late lactation (Noutfia et al., 2014). Decreasing fat, protein, non-fat dry matter, and total dry matter in the composition of milk decreases milk quality (Peana et al., 2007).

In intragroup analysis protein content from day 1 to day 3 (for both primiparous and multiparous groups) did not show statistically significant differences (P>0.9999). When intragroup day 1 and day 7 were compared, statistically significant differences were observed (P-Day 1 vs. P-Day 7: P=0.004 and M-Day 1 vs. M-Day 7: P=0.0008). A similar situation was observed when P-Day 3 vs. P-Day 7 (P= 0.0244) and M-Day 3 vs. M-Day 7 (P<0.0001) were analyzed. The intergroup analysis did not show statistically significant differences (P>0.9999) (Figure 2). Other studies show a similar decrease in total protein content in colostrum and total protein levels from different goat breeds (Graf et al., 1970; Linzell and Peaker, 1974). Other studies show a similar decrease in total protein and fat contents in goat colostrum; fat percentage at postpartum was higher than second day and the following days (Arguello et al., 2006; Sánchez-Macías et al., 2014) and total thus concluding that the nutritional requirements of the newborn goat are significantly higher than the nutritional requirements in the growth and fattening process.

Casein and urea content showed statistically significant differences in all intragroup analyses (P<0.0001) but in intergroup analysis, no significant differences were observed (Figure 2). The high casein content in the first postpartum days is explained by its ability to form a coagulum and facilitate the digestion of the newborn goat, observing a regression until the seventh day, when nutritional requirements are no longer as high (Bergman & Turner, 1937). Milk urea has been used as an indicator of the adequacy of the ratio of crude protein to energy intake in animal nutrition. Milk urea is influenced by milk production, age of animals, number of lactations, stage of lactation, and grazing system (Oltner et al., 1985; Carlsson et al., 1995).

The pH levels in the primiparous group were statistically significant (P-Day 1 vs. P-Day 3; P-Day 1 vs. P-Day 7; P-Day 3 vs. P-Day 7: P<0.0001). In the case of the multiparous group, pH levels were statistically significant only in M-Day 1 vs. M-Day 3 and M-Day 1 vs. M-Day 7 intervals (P<0.0001) while in M-Day 3 vs. M-Day 7 interval, no statistically significant differences were observed (Table 1 and Figure 3).

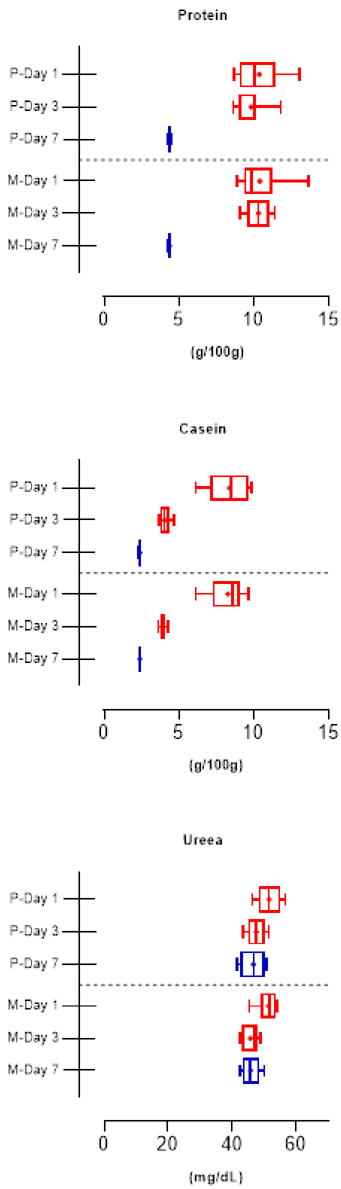

Figure 2. Variations and descriptive statistics of protein, casein, and urea content were determined for the primiparous and multiparous groups. Red Box and whisker plots represent colostrum analysis (day 1 and day 3). Blue box and whisker plots represent milk analysis (day 7). P represents the primiparous group and M represents the multiparous group

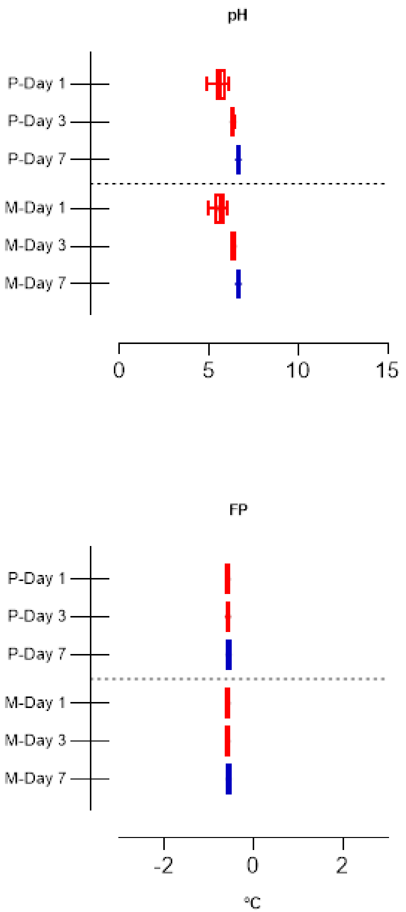

Figure 3. Variations and descriptive pH and FP content were determined for the primiparous and multiparous groups. Red Box and whisker plots represent colostrum analysis (day 1 and day 3). Blue box and whisker plots represent milk analysis (day 7). P represents the primiparous group and M represents the multiparous group

The biochemical components of milk gradually increase from the first lactation to the fourth lactation, except for lactose and pH (Bhosale et al., 2009). pH has a significant effect on the thermal stability of goat milk proteins, and goat milk proteins are the least thermally stable at pH 6.9 (Li et al., 2020).

FP content in the primiparous group did not show statistically significant differences in P-Day 1 vs. P-Day 3 intervals (P>0.9999) while in P-Day 1 vs. P-Day 7 and P-Day 3 vs. P-Day 7 intervals the differences were statistically significant (P=0.0002 respectively P=0.0232). In the multiparous group, FP content showed a similar tendency: M-Day 1 vs. M-Day 3 (P>0.9999), M-Day 1 vs. M-Day 7 (P=0.0025), and M-Day 3 vs. M-Day 7 (P=0.001). The intergroup analysis (primiparous vs. multiparous) did not register significant statistical differences (P>0.9999) (Figure 3). The freezing point (FP) of milk is relatively constant as a consequence of the osmotic balance in milk and blood (Henno et al., 2008), for goat's milk an average reference value of -0.579°C was established (Dharamarajan et al., 1950). Freezing point is significantly influenced by breed, genetic factors (species and breed) as well as non-genetic factors such as feed composition, water intake, milking time, stage of lactation, season, and livestock size (Gencurová et al., 2008; Kędzierska-Matysek et al., 2011).

Bacterial mammary infections are the major cause of increased SCC in dairy goats, but there are a large number of non-infectious factors that can significantly affect SCC in goat milk, such as time between milkings, stage of lactation, number of lactations, and breed (Jiménez-Granado et al., 2014).

The SSC determinations showed significant statistical differences in the primiparous group for all intervals (P-Day 1 vs. P-Day 3: P<0.0001, P-Day 1 vs. P-Day 7: P=0.0006, P-Day 3 vs. P-Day 7: P<0.0001). In the multiparous group statistically, significant differences were observed for M-Day 1 vs. M-Day 3 (P<0.0001) and M-Day 3 vs. M-Day 7 (P<0.0001), while for the interval M-Day 1 vs. M-Day 7, no significant statistical differences were observed (P=0.1145). Intergroup analysis (primiparous vs. multiparous) showed that there were no statistically significant differences (P-Day 1 vs. M-Day 1: P= 0.5499; P-Day 3 vs. M-Day 3: P=0.9964 and P-Day 7 vs. M-Day 7: P>0.9999) (Figure 4). Normal limits of SCC vary between 210 x 1.000 and
1.120 x 1.000 cells/ml in goat milk (Manser, 1986). According to the reference range of SCC in the specialized literature and the results of the analyzes performed on the milk of the Carpathian breed goats, it can be said that the health status of the mammary gland is adequate. In other studies, the lactation stage was shown to have no significant effect on SCC and TBC, and the correlation between these parameters was also non-significant (Kuchtík et al., 2021).

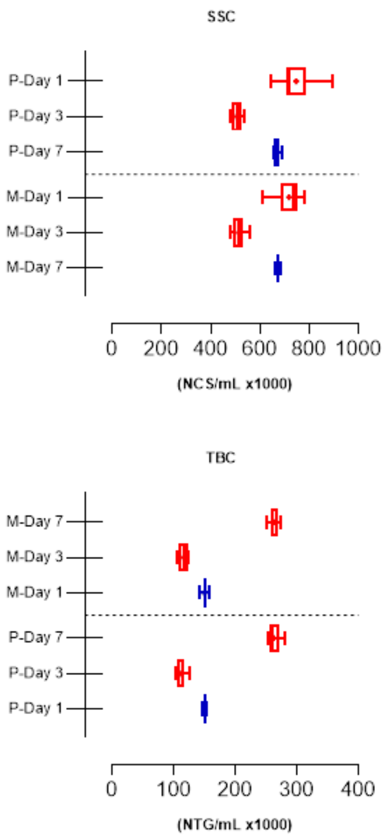

Figure 4. Variations and descriptive statistics of SSC and TBC content determined for primiparous and multiparous group. Red box and whisker plots represent colostrum analysis (day 1 and day 3). Blue box and whisker plots represent milk analysis (day 7). P represents the primiparous group and M represents the multiparous group

TBC is commonly used to assess the bacteriological quality of milk. The goat's milk sampling protocol was strictly followed to obtain the accuracy of the data regarding the bacterial load, so we can state that for the analyzed Carpathian goats TBC was influenced by the period of lactation, but not by their physiological status (primiparous and multiparous). The TBC determinations in both groups (primiparous and multiparous) were statistically significant (P<0.0001). Intergroup (primiparous vs. multiparous) analysis did not show statistically significant differences between groups (P-Day 1 vs. M-Day 1: P>0.9999; P-Day 3 vs. M-Day 3: P=0.4856 and P-Day 7 vs. M-Day 7: P=0.9855) (Figure 4). Many studies show that the bacteriological quality of goat milk is mainly influenced by the milking method, water quality, hygiene of milking equipment and storage tanks, hygiene of milkers, milking parlor environment, and transport hygiene (Contreras et al., 2003).

CONCLUSIONS

The physiological status can produce significant changes in the physical-chemical parameters of the milk of Carpathian goats raised in the area of the Trascău Mountains, Alba County, Romania. Changes in Fat, Lactose, and SNF content registered identical trends, with intragroup analysis showing statistically significant differences only when comparing day 1 to day 7, while the intergroup analysis did not show statistically significant differences. Protein content from day 1 to day 3 (for both primiparous and multiparous groups) did not show statistically significant differences but observed statistically significant differences intragroup day 1 and day 7. A similar situation was observed from primiparous Day 3 vs. Day 7. Casein and urea content showed statistically significant differences in all intragroup analyses but in intergroup analysis, no significant differences were observed. The casein and urea content in Carpathian goat milk was influenced by the stage of lactation. FP content in the primiparous group did not show statistically significant differences and in the multiparous group, FP content showed a similar tendency. This tendency shows that the freezing point of milk is relatively constant. The pH levels were statistically significant in the primiparous and multiparous groups, but intergroup there were no statistically significant differences observed. Protein, Casein, SNF, and TS were observed to decrease throughout the seven days studied, except for lactose which showed an increase. The composition of the goat's milk was different in the first three days compared to the parameters of normal milk, which demonstrates the power of the milk to support the nutritional needs of the newborn goat kid. The practical applicability of these data can be translated into milk formula recipes adapted for the Carpathian goat biological requirements.

The stage of lactation had no significant effect on SCC and TB, and the correlation between these traits was also insignificant. The SSC determination and the TBC determinations showed significant statistical differences in both groups (primiparous and multiparous). Intergroup (primiparous vs. multiparous) analysis did not show statistically significant differences. According to the reference range of SCC in the specialized literature and the results of the analyzes performed on the milk of the Carpathian breed goats, it can be said that the health status of the mammary gland is adequate.

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DYNAMICS AND FORECASTING OF GOATS ARTIFICIAL INSEMINATION PROCEDURES IN ROMANIA

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Abstract

The efficient breeding of dairy goats depends on the quality of the breeds and the number of high-producing animals, but it also depends to a large extent on the fertility of the goats, their ability to reproduce offspring with normal reproductive functions. Although goat research worldwide is older and more developed than in Romania, information on this species has been limited for a long time, as most research institutes have presented their results jointly for small ruminants, especially sheep and goats. Artificial insemination (AI) is an assisted reproductive technology that involves the manual placement of semen into the female's reproductive tract through means other than natural mating. This method facilitates the meeting of gametes, namely sperm and oocytes, resulting in the formation of offspring. The conventional method for artificial insemination (AI) in sheep and goats in Romania involves the use of fresh or chilled semen, which has provided satisfactory fertility outcomes. The implementation of genetic enhancement techniques and the utilization of imported semen that meets safety standards, necessitating the use of cryopreservation, have the potential to significantly improve milk yield. The objective of this study was to examine the dynamics of artificial insemination (AI) in goats within Romania and to generate a short-term forecast regarding the utilization of this biotechnology in the country's farming conditions. The analysis of the forecast data indicates a positive trajectory in the quantity of AI within the time frame of 2022 to 2026. Specifically, it is anticipated that the number of AI will rise from 2956 in 2023 to 3603 in 2026, signifying an approximate 18% increase. The upper confidence limit suggests that the range of AI could potentially reach between 4310 in 2023 and 6311 in 2026.

***Key words**: artificial insemination, efficiency, forecast, goats.*

INTRODUCTION

The significance of goats in various regions of Europe, particularly in hilly and mountainous terrains, as well as in remote, marginalized, and semi-arid regions, is noteworthy (Lu, 2019; Haenlein, 2017). Goats have the ability to acclimate to diverse agricultural methodologies, environmental circumstances, and topographical landscapes, thereby utilizing substandard resources and converting them into superior products. Goats hold significant cultural importance in various festivals and celebrations, including "Kukeri" in Bulgaria and "Capra" in Romania, as well as other customary folk practices. Dairy goats in Europe serve a multitude of purposes, as outlined by Ribeiro (2010). These include their primary function at the farm level, which involves the production of milk and meat products. Additionally, they serve a secondary function within the industry or supply chain by supplying and processing dairy and meat products. Furthermore, they serve various tertiary functions, such as having a socio-cultural impact on the rural community, maintaining the balance of the land, enhancing landscape aesthetics, providing nutritional value, ensuring food security, facilitating hunting, promoting tourism, and aiding in fire protection.

The majority of goat farming systems prioritizes meat production, accounting for approximately 80% of the industry. However, in developed nations, there is a greater emphasis on dairy goat production and the production of fibers such as cashmere and mohair. The breeding programs for dairy goats that are most advanced are situated in France and are founded on a robust market for goat cheese. Caprigene France conducts selection programs for the Saanen and Alpine breeds, utilizing AI and milk recording. The program encompasses 300000 animals across 2500 herds, which are registered for their milk characteristics. According to Moore's (2017) findings, dairy goat production is also present on a smaller scale in Italy, Norway, and Spain, with a limited number of animals registered in other nations. The primary characteristics pertaining to milk production in goats include the overall milk yield and the protein and fat content of the milk (Popica, 2015; Priseceanu, 2015). Sex-limited traits, which are only measurable after the production of the first offspring, could potentially be improved through the implementation of marker-assisted selection techniques. The production of goat meat is prevalent in various developing nations, albeit with limited noteworthy breeding initiatives. In Australia and South Africa, genetic assessment is being conducted for Boer and other meat breeds of goats, wherein the primary trait evaluated is typically the weaning weight. The utilization of ultrasound technology for assessing fat and muscle traits in goats is not as prevalent compared to other applications. Additionally, reproductive traits in goats have not been extensively studied, likely attributed to their low heritability and the fact that goats are multiparous in nature.

The effectiveness of dairy goat breeding is contingent upon the quality of the breeds and the quantity of animals that exhibit high productivity. The extent to which goats are able to reproduce offspring with normal reproductive functions, known as fertility is a significant determining factor. The capacity in question is influenced by a variety of factors, including environmental conditions, nutritional intake, breeding techniques, and breeding proficiency, as noted by Miao (2016). The functional trait of goat fertility holds significant importance as it stands as a primary selection criteria for small ruminants, as stated by Malher X. in 2001. The enhancement of goat fertility has been a significant focus within the Asian goat industry. Various techniques, including crossbreeding, feed management, oestrus control, artificial insemination (AI), and transcervical embryo transfer (TE), have been employed to achieve this objective. As a result, notable economic gains have been realized (Miao, 2016). Insufficient investigation and analysis in the fields of reproductive physiology and fertility control have resulted in unfavourable breeding outcomes being attributed to an inadequate and short-sighted breeding strategy. The implementation of heat synchronization techniques has been extensively employed in inducing cycles in dairy goats during non-breeding seasons and in synchronizing oestrus, resulting in the standardization of calving intervals and lactation periods (Holtz, 2005; Arredondo, 2015). According to Rahman (2008), the implementation of suitable synchronization protocols can have a notable impact on the timing, frequency, and length of oestrus, as well as the rates of gestation and calving. According to Pendleton (1992), the dairy goat industry commonly employs PMSG + CIDR or FSH + CIDR protocols as efficacious methods for synchronization or superovulation. Drion P.V. (1992) reported a decrease in reproductive performance among female subjects subjected to repeated gonadotropin treatment in farming practices. Research has demonstrated that excessive repetition of superovulation through the use of PMSG or FSH can result in negative effects on both ovarian development and reproductive performance. According to Swanson (1996) and Combelles (2003), repeated induction of superovulation in an animal can lead to a decrease in treatment response and gamete quality. The occurrence of reduced fertility rates subsequent to repeated gonadotropin treatments has been associated with an elevation in anti-FSH (Baril, 1992) or anti-PMSG (Swanson, 1996) antibodies. There is speculation that the administration of multiple treatments may trigger an immune response that effectively neutralizes the biological activity of gonadotropins. In animals, elevated levels of anti-PMSG or anti-

FSH antibodies have been linked to ovarian refractoriness, as noted by Bavister (1986) and Remy (1991). The utilization of artificial insemination (AI) in sheep and goats has conventionally involved the use of fresh or chilled semen, yielding satisfactory fertility outcomes. The utilization of exotic breeds, genetic enhancement, and safe' semen from foreign nations requires the utilization of cryopreserved semen to enable the examination of pollutants or illnesses in the 'donor' male before the administration of semen doses for artificial insemination. The acceptability of post-thaw motility of frozen semen from goats and sheep is generally acknowledged; however, its utilization in artificial insemination (AI) has been linked to reduced fertility, primarily attributable to a shorter lifespan of sperm.

MATERIALS AND METHODS

The objective of this study was to examine the AI trends in the Romanian goat industry and generate a projection regarding the growth trajectory of this market segment at a national level.

The examination of the AI dynamics in Romania and the formulation of a brief and intermediate-term projection of AI utilization were conducted utilizing information sourced from the records of various entities with the consent of their proprietors, facilitated by the ANCC-CAPRIROM Association and ANARZ. ANCC-CAPRIROM is the accredited association for the management of the herd book of most breeds bred in the country and to carry out production control on these goats. Since 2000, it is affiliated to the International Goat Association (IGA, 2019 www.goatworld.org). Artificial insemination activities in goats in Romania are carried out only through this association.

The research employed a dynamic or time series methodology to account for the variability of data on the dynamics of AI in the studied species. The methodology involved the use of fixed-base forecast indicators calculated through the application of the exponential smoothing method.

Dynamic/time series highlight the temporal character of phenomena and are an important tool in the context of macroeconomic analyses. Specific to dynamic series is that they are defined for complex entities, characterised by a high level of variation in indicator data, including structural time variations to which they are subject and the actions involved in this study (AI). The statistical analysis of the data utilized in the current study was carried out through Microsoft's Analysis ToolPak and Microsoft Excel 2016 software. Furthermore, the statistical interpretation of each graph was completed within the context of the results obtained. Regression analysis was conducted on each dynamic series, given that it is a well-established model in the field of numerical simulation. This model aids in the interpretation of algorithms that have practical applications in various fields, including but not limited to advertising, medical research, and agricultural sciences. The interpretation of R^2 in linear regression models pertains to its function as a measure of goodness of fit. The assessment considers the degree of correlation between the model and the dependent variable. The range of the limit is between 0 and 100 %. The utilization of regression analysis facilitated the examination of the association between the independent variables, also known as explanatory variables, and the dependent variables, also known as response variables. This facilitated the assessment of the proximity between the approximated value and the actual value. The regression equation was generated utilizing the Analysis ToolPak technique, wherein statistical parameters are utilized to represent the relationship between the explanatory variable and the response variable. The R^2 coefficient is employed to examine the extent to which variations in one variable can be accounted for by variations in a second variable. R^2 indicates the extent to which the independent variable accounts for the variation in the dependent variable. The interval spans from zero to one, indicating that the variable x can account for up to 100% of the variability in y.

RESULTS AND DISCUSSIONS

Results and discussion on AI dynamics per total goat herd in Romania

The total number of AI in the total goat herd of our country during 2010-2022 was 25908. Their distribution by years is shown in Figure

1. An increase in the number of AI doses, i.e. number of AIs in goats in the period 2010-2019, is observed from 260 in 2010, a doubling of this number in 2011, then an increase of 16.6% to 88% in the period 2011 - 2019. This is followed by a decrease of 38.9% during 2019-2020 and about 14% in 2020-2021. In 2022 there was an increase of about 29%. We explain this decrease in the number of AIs against the background of the COVID-19 pandemic, which has affected, as we know, all socio-human activities and probably also this sector.

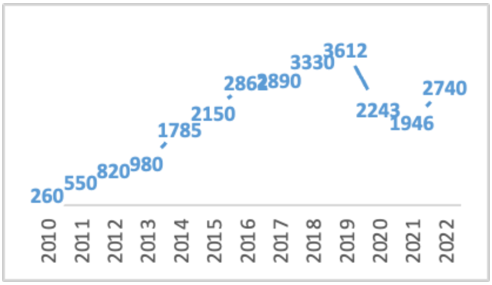

Figure 1. Dynamics of the AIs number in Romania during 2010-2022

Carpathian breed AI dynamics results and discussion

As known from INSSE data, the Carpathian breed is mostly spread in the lowland areas and represents about 85-90% of the total goat herd raised in the country. The ancestor of the Carpathian breed is the Prisca goat and is characterised by a great variability in coat colour, body conformation and productivity. The notable heterogeneity observed in this species can be attributed to the absence of systematic breeding practices, whereby the species has undergone self-breeding and improvement solely through the indigenous knowledge of the breeders. The objective of the genetic resource conservation and utilization initiative for the Carpathian goat breed is to enhance the breed's genetic makeup through biotechnological breeding techniques, such as artificial insemination. According to data provided by ANCC-CAPRIROM and ANARZ, Figure 2 illustrates the frequency of artificial insemination procedures conducted within this particular breed from 2010 to 2022. The data indicates a rise in the quantity of artificial insemination commencing in 2010 and persisting until 2019, followed by a decline of approximately 40% in 2020 and 30% in 2021. The current year has witnessed a rise of approximately 35% in the aggregate count of artificial insemination (AI) within this particular breed.

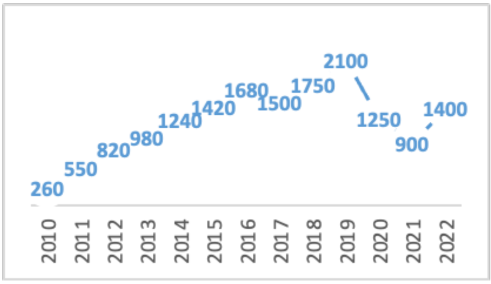

Figure 2. Dynamics of the AI number in the Carpathian breed during 2010 and 2022

French Alpine breed AI dynamics results and discussion

The integration of our country into the European Union has meant the opening up of a vast market for agri-food products, characterised by high absorption potential, high purchasing power and relative price stability. The application of modern goat breeding systems has meant supplementing the traditional methods used to improve genetic potential with methods for assessing the herd base and their productive capacity so that faster and more efficient decisions can be made in the selection and breeding process. Thus, French Alpine goats are included in the national breeding programme, with more than 3900 females included so far. Figure 3 shows the number of AI achieved in females of this breed since 2014. An upward trend was observed during the period spanning from 2014 to 2016, which was followed by a modest decline from 2018 to 2021, amounting to a reduction of 12.5%. Subsequently, there was a rise of approximately 7% in 2022. At the current stage of improvement, in the French Alpine breed where the availability of purebred males is low, there is a ranking of females distributed in a decreasing way to purebred males also ranked. Caprirom's recommendation is to use artificial insemination with semen from breeding males. By changing males every 4-5 years or using artificial insemination, inbreeding at farm level is also avoided. Therefore, following the ranking of females based on the breeding value of milk production and the ranking of males based on the breeding value of the dam, ANCC-CAPRIROM is the company that recommends the breeding system for goat farms in the herd book and suggests an increase in the number of AI in this breed.

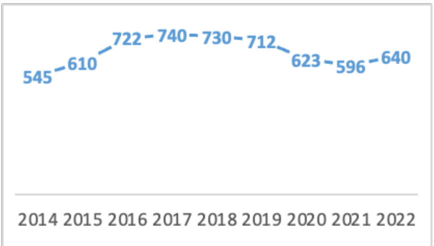

Figure 3. Dynamics of the AI number in the French Alpine breed during 2014 and 2022

Results and discussion on AI dynamics in the Saanen breed

As in the case of the French Alpine breed, in Romania, the Swiss-breed, Saanen is included in the goat herd improvement programme and participates with a herd of more than 4600 females and 250 goats and calves. Figure 4 details the number of AI carried out between 2015 and 2022. It shows an increase in the number of AI from 2015 up to and including 2018, from only 120 doses to 850 doses, followed by a decrease of more than 50% in 2020. The situation has recovered, with an increase of up to 52.8% in 2022.

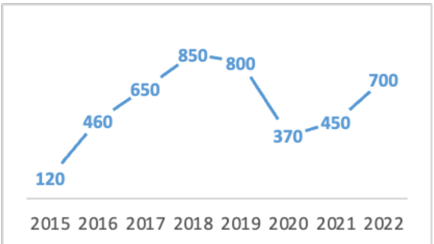

Figure 4. Dynamics of the AI number in the Saanen breed during 2015 and 2022

According to ANCC-CAPRIROM's analysis of the dynamics of the number of AIs by breed in Romania, the Carpathian breed recorded the highest number of AIs, rising from 260 in 2010 to 2100 in 2019. Regarding the French Alpine breed that was selectively bred in Romania, the initial artificial insemination report was documented in 2014, with a cumulative count of 545 AI procedures conducted throughout the year. Subsequent to an initial rise, there was a subsequent peak in 2017, wherein a cumulative sum of 740 AI was documented, followed by a gradual decline culminating in 596 AI doses in 2021. By December 1st of 2022, there had been a notable advancement in the field, with a total of 640 inoculated females. The initial documentation of the Saanen breed, which was selectively bred in Romania, dates back to 2015, specifically with regards to 120 artificial inseminations. Subsequent to an exponential surge, the number of artificial inseminations recorded in 2018 amounted to 850. This number remained stable in the year following but experienced a marked decline in 2020, with only 370 AI cases being recorded. However, in the subsequent two years, there was an upward trajectory, culminating in 700 AI cases being recorded. Upon analysis of AI number in goats in Romania, a discernible trend emerges: a decline in AI number during the period of 2019-2020. This trend is likely attributable to the global situation resulting from the COVID-19 pandemic, which has had a detrimental effect on the livestock service sectors of numerous countries. This finding serves to reaffirm the idea that external factors, specifically those pertaining to the social environment, have the potential to cause disturbances in the execution of economic service operations. Globally, artificial insemination stands as the sole biotechnical approach accessible to breeders for enhancing production efficiency in goats. The key prerequisite for attaining this favourable outcome is the identification of goats with the greatest breeding potential, whose genetic advancements can be disseminated across goat farms over time and space.

Results and discussion on AI forecasting in goats in Romania

In Romania, although there are national goat breed improvement programmes and the number of goat holdings exploited for milk is high and at the same time the number of goats, contrary to our expectations, the AI situation in goats has not improved in a remarkable way, as overall the fecundity by using AI in this species does not exceed the value obtained by natural service and at the same time the milk production recorded in official control does not seem to excel. Improvement programmes for dairy goats also include the use of natural service systems, which achieve fecundity indices above 85%.

Results and discussion on AI forecasting per total goat herd in Romania

The situation of the forecast of the total goat herd at national level where AI was used is shown in Figure 5.

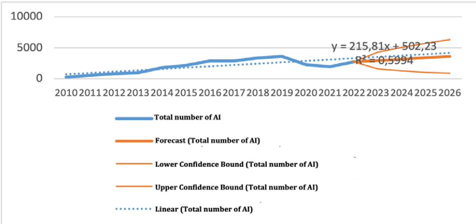

Figure 5. Short-term forecast of the number of AIs in Romania

The graphical representation of the regression equation indicates a positive trend in the number of AIs from 2022 to 2026, with a coefficient of determination of approximately 60%, suggesting a likelihood of increased AIs. The forecast calculations indicate an anticipated rise in the number of AIs from 2956 in 2023 to 3603 in 2026, signifying a growth of nearly 18%. The range of the projected values lies between 4310 AIs in 2023 and 6311 AIs in 2026, as presented in Table 1.

Table 1. Forecasted number of AIs in Romania for 2023-2026

Year	AI estimated number	Lower confidence bound	Upper confidence bound
2023	2.956	1.602	4.310
2024	3.172	1.258	5.085
2025	3.387	1.043	5.731
2026	3.603	896	6.311

Results and discussion on AI forecast for the Carpathian breed

The Carpathian breed, representing almost 78% of the national goat population, was used for AI by the breeding programme proposed by ANCC-CAPRIROM. Based on the data recorded by this association, Figure 6 shows the dynamics and the short-term AI forecast for this breed. Based on the regression equation, the forecast values for the number of artificial insemination in this breed for the period 2022 - 2026 were calculated (Table 2). The trend shown graphically is upward, with values ranging from 1481 AI in 2023 to 1725 AI in 2026. In the same sense, taking into account the coefficient of determination of 58%, we can estimate higher values for the mentioned interval, namely 2378 AI in 2023 and 3198 AI in 2026.

Table 2. Number of AIs in the Carpathian breed forecast for the period 2023-2026

Year	AI estimated number	Lower confidence bound	Upper confidence bound
2023	1.481	584	2.378
2024	1.562	440	2.684
2025	1.643	334	2.952
2026	1.725	252	3.198

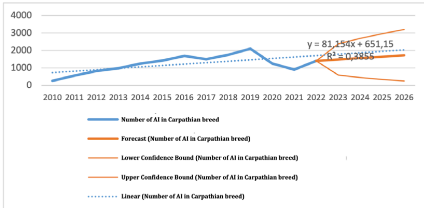

Figure 6. Short-term forecast of the number of AI in the Carpathian breed

Results and discussion on AI forecast for Alpine breed

The French Alpine breed has been imported into our country from Eastern European countries during the last 12 years. Due to improper exploitation, i.e. not respecting the feeding technology, milking rhythm and maintenance conditions, the goat herds have registered extremely low values, although it is included in the clean breed improvement program, in order to increase milk production and to achieve characteristic performances in the breeding and climate conditions specific to our country. The graphical representation depicted in Figure 7 presents a short-term projection of the expected number of artificial insemination procedures to be conducted on the French Alpine breed within Romania in the near future. Table 3 illustrates a modest upward trajectory, wherein the mean values fluctuate between 644 AI in 2023 and 655 AI in 2026, indicating a yearly growth rate of merely 4-6%. By utilizing the regression equation, it is possible to make an estimation of the attainable quantity of artificial inseminations under optimal circumstances, specifically 915 AI by the year 2026.

Table 3. Number of AI in the French Alpine breed forecast for the period 2023-2026

Year	AI estimated number	Lower confidence bound	Upper confidence bound
2023	644	531	757
2024	647	480	815
2025	651	436	866
2026	655	395	915

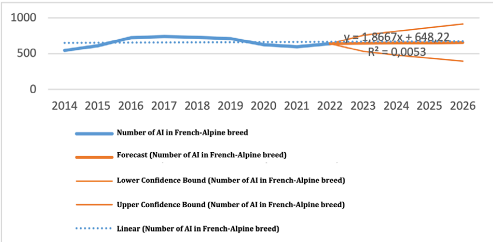

Figure 7. Short-term forecast of the number of AI in Romania in the French Alpine breed

Results and discussion on AI forecast for the Saanen breed

The breeding programme of the Saanen breed in Romania aims to achieve the performance characteristic of the breed in the country of origin, under the specific growing and climatic conditions of our country, i.e. to improve milk production characteristics. The breed is very robust and hardy and has adapted well to different climatic and feeding conditions. The breed is sensitive to sunlight and is more productive if kept in cooler premises. The Saanen breed goat is easy to maintain and can be reared very well in intensive systems for milk production, with good adaptability to mechanic milking and environmental conditions. Chart 8 depicts the trend of artificial insemination (AI) utilization within this particular breed in Romania, indicating a modest upward trajectory in the forthcoming period. Table 4 presents the anticipated numerical values for artificial intelligence during the timeframe spanning from 2023 to 2026.

Table 4. Numbers of Saanen breed AIs forecast for 2023 - 2026

Year	AI estimated number	Lower confidence bound	Upper confidence bound
2023	716	260	1.172
2024	754	140	1.368
2025	791	52	1.530
2026	829	-17	1.675

Based on the confidence interval analysis, it can be inferred that the estimated count of AI in the Saanen breed in Romania falls within the range of 261 to 1175. It is noteworthy that this range may indicate a marginal decline in the count, subject to certain conditions.

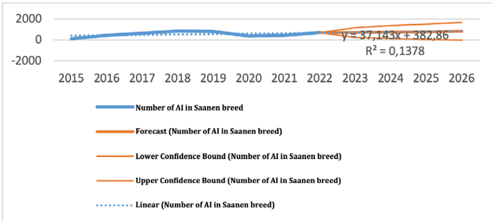

Figure 8. Short-term forecast of the number of AI in the Saanen breed

CONCLUSIONS

The general trend in Romania regarding the total number of AIs has exhibited an upward trajectory from 2010 to 2019, with numbers rising from 260 AIs in 2010 to 3612 AIs in 2019. However, there was a subsequent decline in the years 2020 and 2021, with 2243 AIs and 1946 AIs, respectively. Nonetheless, there was a subsequent increase of approximately 29% in 2022, with 2740 AIs. Upon analysis of the AI population dynamics across various breeds in Romania, it is evident that the Carpathian breed has consistently maintained the highest number of AIs. This trend is reflected in the data reported by ANCC-CAPRIROM, which indicates that the number of AIs for this breed has increased from 260 in 2010 to 2100 in 2019. Regarding the French Alpine breed in Romania, the initial artificial insemination (AI) report was documented in 2014, with a cumulative count of 545 AI procedures performed throughout the year. Following an initial rise, there was a subsequent peak in 2017, with a cumulative count of 740 AI. This was then followed by a modest decline, culminating in a total of 596 AI doses in 2021. By December 1st, 2022, there had been notable advancements in the field, with a total of 640 inoculated females. The initial documentation of the Saanen breed in Romania dates back to 2015, when 120 artificial insemination procedures were conducted. Subsequent to an exponential surge, the number of artificial insemination (AI) cases escalated to 850 in 2018 and then stabilized at a plateau in the subsequent year. However, a marked decline was observed in 2020, with only 370 AI cases being recorded. In the ensuing two years, there was an upward trajectory, culminating in the attainment of 700 AI instances. Upon analyzing the dynamics of artificial insemination (AI) numbers in the goat population of Romania, a salient trend emerges: a decline in AI numbers during 2019-2020. This phenomenon is plausibly attributable to the global situation stemming from the COVID-19 pandemic, which has adversely affected the livestock service industries in numerous nations. This finding serves as further evidence that external factors, specifically those pertaining to the social environment, have the potential to cause disturbances in the execution of economic service operations. The analysis of the forecast indicates an upward trajectory in the quantity of AIs during the period spanning from 2022 to 2026. It is projected that the number of AIs will rise from 2956 in 2023 to 3603 in 2026, reflecting an increase of nearly 18%. The upper confidence limit suggests that the range of AIs could be between 4310 in 2023 and 6311 in 2026. According to the projected estimates, the Carpathian breed exhibits a discernible upward trend, with mean values ranging from 1481 AI in 2023 to 1725 AI in 2026. Additionally, there is a possibility of surpassing this figure up to 3198 AI in 2026, as indicated by the upper confidence limit. The French Alpine breed exhibits a modest positive trend, with mean values ranging from 644 AI in 2023 to 655 AI in 2026, indicating an annual growth rate of merely 4-6%. There is a likelihood of a moderate increase in the population of Saanen AIs in Romania in the foreseeable future. Based on the confidence interval analysis, the estimated range for the number of artificial inseminations (AI) in the Saanen breed in Romania falls between 261 and 1175. This range may indicate a slight downward trend, as suggested by the upper, middle, and lower confidence limits. While goat production may not be a globally significant sub-sector of livestock in terms of economic value, it holds significant environmental and social importance. Therefore, it is a strategic sector that warrants maintenance and improvement. Despite the increasing prevalence and intensification of European goat farming, there remain numerous regions where goats maintain significant connections with the surrounding environment, thereby producing valuable ecosystem services. Further enhancements are required to the facets that can render this undertaking lucrative and appealing to the youth demographic, with the aim of increasing recognition of the worth of goat-derived products and securing corresponding remuneration. Additionally, it is imperative to acknowledge and appreciate the societal and environmental contributions of goat farming, particularly in underprivileged rural regions. In summary, it can be asserted that information regarding the efficacy of artificial insemination in this particular animal species, its patterns of behaviour, and a forecast of its utilization in the near and intermediate future would be highly beneficial in devising tactics to enhance the production of milk and meat in this species.

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INFLUENCE OF TEMPERAMENT ON MATERNAL BEHAVIOUR IN DAIRY GOATS

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Abstract

The behaviour of the mother goat plays a decisive role in the survival of the newborn kid. There are conflicting opinions regarding the influence of temperament on maternal behaviour in different animal species. In goats, as species that possess a behaviour of "hiding" their newborn, temperament should significantly influence the development of the relationship between the newborn kid and its mother. This is especially important in animals giving birth for the first time. Knowing the relationship between temperament and maternal response in goats would lead to better management of technological processes on the farm, which in turn would increase kid survival rates and reduce mortality rates. Various tests are used worldwide to determine the temperament of farm animals, some of which are applicable to goats. The aim of this review is to summarize the methods of determining temperament in dairy goats and how it affects the goat's behaviour towards the kid.

Key words: *goats, kids, maternal behaviour, temperament.*

INTRODUCTION

The change in emotional reactivity of animals represents a phenotypic trait that has been called a temperament (Blache & Bickell, 2010). Individual differences in a temperament may influence the way an animal responds to new situations, forages, counters predator threats, and patterns its sexual behavior (Réale et al., 2000; 2007). According to Plush et al. (2011), the temperament can be used as a tool to improve the quality and to increase the productivity of animal production.

Data in the available literature on the relationship between goat temperament and maternal behavior is not enough. Although the mother-newborn spatial relationship in sheep (following) and goats (hiding) is different, the maternal behavior in both species is very similar, as sensory and physiological control is identical in almost all aspects (Hernández et al., 2012). In most studies, sheep and goats are placed under the same denominator. For this reason, this paper looks at the temperament and maternal behaviour of the sheep.

The aim of this review is to summarize some of the methods of determining temperament in dairy goats and how it affects the goat's behavior towards the goat kid.

MATERIALS AND METHODS

This review is based on the analysis of the available bibliographic sources and summarizes some of the temperament determination methods that can be used in dairy goat farming. The relationship of temperament to maternal behavior was also examined.

RESULTS AND DISCUSSIONS

Studies, which assess temperament and its potential relationship to maternal behaviour in sheep, have focused on measuring fear responses and individual variation in behavioural reactivity to different situations (Dwyer, 2013). The behavior patterns, which have been induced by fear, change depending on the potential threat (Forkman et al., 2007).

Forkman et al. (2007) summarized tests to determine fear susceptibility in sheep and goats. According to them, the most commonly used test is the Novel arena test, which is usually conducted in an arena with a size of 4 m x 4 m, for about 5 min. and a different number of variables are observed (activity, vocalizations, etc.). In second place, they do the Novel object test, which measures reactions to an unfamiliar new object, and lastly, Restraint and human fear tests, which measure the animal's reaction to a person (a stationary and moving one).

Most of the tests involve direct observation of animals, which is time-consuming and prone to errors. For this reason, modern technologies based on video surveillance and the use of different types of sensors are being developed (Bati & Ser, 2023).

According to Dwyer (2013), in studies investigating maternal behavior, although there seems to be a relationship between ewe temperament and maternal behaviour, in fact the picture is not very clear and how this behavior is governed has not been realised entirely yet.

Tests for the evaluation of temperament in sheep and goats

Lyons et al. (1988) used an Arena test to evaluate the behavior of alpine dairy goats of different ages towards humans.

Németh et al. (2009) studied the temperament of three goat breeds, such as Sanental, Alpine and Selected Hungarian. They conducted a balance test according to Trillat et al. (2000). Animals kept the balance for up to 30 seconds during the test, while they are scored according to their behaviour on a scale of 1 to 5, according to the following:

1. point: calm, not moving;
2. points: calm, some potential movements;
3. points: calm, very little movement, but the balance is not shaken with it;
4. points: sudden, episodic movements, but the balance is not shaken with it;
5. points: continuous, sudden movements, the balance is shaken with it.

According to the authors, this test can be used in goats to evaluate their temperament. They found that temperament changes with age and younger goats are more temperamental than old ones.

Lyngwa (2012) observed fear response and social interactions in pregnant Norwegian diary breed housed at barns with three different types of density of goats. They apply three kinds of tests: a separation test, a human approach test and a social behavior within the herd. The authors reported that no clear conclusion can be drawn about fear responses in goats using these tests because they are cross-sectional, but increased density enhanced the fear response in the animals tested.

Stoycheva et al. (2014) investigated the influence of temperament on the milk productivity of goats of the Bulgarian White Dairy breed and its crossings with the Anglo-Nubian and Toggenburg breeds (Figure 1), applying a modified Lankin test. The authors found that the emotional sensitivity of the goats affected their milk productivity, so that goats with a calm temperament surpassed those with a nervous temperament in terms of milk production.

Figure 1. Goats in milking parlour

Finkemeier et al. (2019) used Open-field and novel-object test, Social separation test for Assessment of personality types in Nigerian dwarf goats (*Capra hircus*) and cross-context correlations to behavioural and physiological responses and found that individual differences in personality in goats are consistent over time and describe the dependencies between behaviour in different test situations and some physiological parameters.

Elmetwally et al. (2021) investigated the effects of maternal temperament on uterine blood flow, fetal heart rate, gestation length, and fetal birth weight in an experimental goat model. The authors applied the Arena test (Kilgour & Szantar-Coddington, 1997) and divided the animals into calm and nervous. After the goats were inseminated, they observed these parameters and found that the mother's temperament had a negative impact on uterine artery Doppler indices, fetal growth and gestation length in an experimental goat model.

The tests described so far are a very small share of all tests and their variations, but they provide a general idea of the main ways of testing.

Assessing the fear susceptibility of animals is very important in relation to humane treatment towards them, which ensures adequate "behavioural relationships" (Mellor et al., 2020).

The application of temperament assessment tests to examine its relationship to maternal behavior (Figure 1) has produced conflicting results.

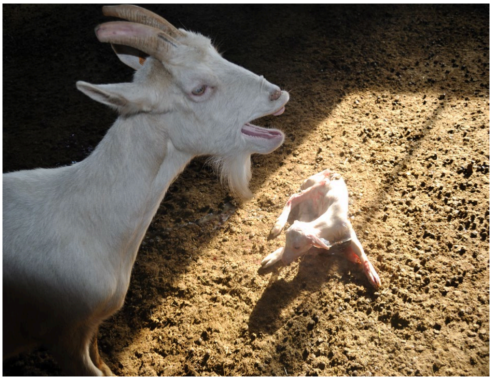

Figure 2. Maternal behavior of goats of the Bulgarian White Dairy breed

Goats as species, and dairy goats in particular, show great flexibility when are faced with changes in the nutritional and environmental conditions, as well as under dynamic social conditions (Zobel & Nawroth, 2020).

The level of maternal care is an adaptive behaviour to the environment with which the individual interacts, in a way that the mother modulates the responsiveness of the offspring to specific early environmental conditions (Summarized by Núnez-Murrieta et al., 2021). The goat mother-kid relationship is a process that involves physiological, anatomical, hormonal, and ethological factors, the alteration of which can lead to risks for the survival of the newborns (Mandal et al., 2022).

The onset of maternal behavior immediately after birth is largely controlled by sex hormones (summarized by Bickell et al., 2011; Bridges, 2015). Hereditability also plays a determining role in the maternal behavior. The combination of these factors leads to the development of individual differences in the temperament of domestic dairy goats (Lyons et al., 1988).

Temperament in Merino sheep is determined primarily by the genetic transmission of the trait rather than behaviour learned from the dam (Blache & Bickell, 2010). Selection for calm temperament in sheep can support lamb survival by improving the maternal behaviour of mothers (Bickell et al., 2010). The authors investigate the extent to which behavioural selection influences the maternal behaviour of sheep after parturition with minimal human intervention. The observations covered the mothers' behaviour two hours after birth and found that calm mothers licked their babies longer and tended to spend more time with them. It has been shown that there is no difference in suckling duration of lambs born to calm and nervous mothers (Bickell et al., 2010). Although the time spent suckling was the same, the amount of nutrients ingested may be different because colostrum from calm-tempered ewes is less viscous than that of nervous ewes and can be sucked more easily (Hart et al., 2006 in Blache & Bickell, 2010).

Emotional sensitivity (temperament) has a great influence on the expression of maternal qualities and especially for defining the selective mother-offspring relationship (Peeva, 2009a; Dimitrov et al., 2009). The degree of performance of maternal behaviour is determined by a series of criteria for evaluating the acceptance or rejection of the newborn (Peeva et al., 2005). Inexperienced mothers (those giving birth for the first time) more often exhibit atypical behaviour towards newborn goat kids compared to multiparous ones (Yılmaz et al., 2012).

Karaca et al. (2016) investigated the relationship between temperament and maternal behaviour score in Saanen x Hair Goat Crossbred Does using arena and isolation box tests. Hierarchical cluster analysis was performed to establish temperamental classes such as calm or nervous. Maternal behaviour was scored based on the proximity of the goat while her kid was marked. The authors found that there was no significant relationship between temperament and ratings of maternal behavior. According to them, further studies are needed to clarify the relationship between temperament and maternal ability, rather than using only assessment of maternal behavior.

Peeva et al. (2005) and Peeva (2005) observed the behaviour of dairy sheep with different types of emotional sensitivity during the first three days after birth. Based on a rank score, they divide the sheep into three types of temperament: calm, nervous and intermediate type. Behavioural reactions were recorded: licking, sniffing, suckling, etc. The calm animals showed the most stable maternal qualities on the first day, which was maintained until the third day. The tendency for increased anxiety persisted until the third day in sheep of nervous type. The authors found that the behavioural indicator of frequency of reaction change is one of the most characteristic for distinguishing the type of emotional sensitivity during the first 24 hours after parturition. Repeating the same experiment (Ivanov et al., 2005) confirms that animals with a calm temperament have the most stable maternal qualities.

Peeva (2009b) studied the influence of temperament on the maternal behaviour of primiparous dairy sheep by applying three tests: assessment of temperament in the milking parlour according to Dimitrov et al. (2009); a system of fear inducing and learning tests according to Dimitrov et al. (2009). Based on the combined score from these tests, the temperament of the sheep is divided into calm, nervous and average type. The "Mother - young separation I" Test (visual and tactile contact allowed) was used to assess the influence of temperament on maternal behaviour. The author demonstrates that sheep with a nervous temperament show hesitation when approaching the separated lamb compared to those with a calm temperament.

There is evidence that lambs born to calm dams have a better chance of survival between birth and weaning (Murphy et al., 1994; Murphy, 1999 in Blache & Bickell, 2010). According to the authors, the lamb mortality of calm dams is lower than that of nervous sheep because calm ones spend more time with their lambs, move a shorter distance, and return to them more quickly (summarized by Blache & Bickell, 2010).

Gender differences in the expression of maternal behaviour have been found (Dwyer & Lawrence, 2005).

Von Borstel et al. (2011) found such a difference in different breeds of sheep raised in the same conditions, feeding regime and management. According to them, the genetic selection of animals with desirable maternal behaviour seems possible.

CONCLUSIONS

Despite the satisfactory results reported by all researchers regarding the dependence of maternal behaviour on the animal's temperament, it is still not fully known how this process occurs. Scientists are aware that this is a process that is influenced by the interaction of a number of factors. But so far, no exact answer has been given to the question, which of them is the most important and is there one? Compared to other animal species, the goat has not yet been well studied regarding the relationship between temperament and maternal behaviour. It is possible that the influence of temperament in goats on maternal behaviour is most pronounced at critical moments. Knowing these dependencies will lead to better management of technological processes on the farm, which in turn would increase survival rates of goat kids and reduce mortality rates.

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OPTIMATION OF NONI FRUIT EXTRACT USING ZINC OXIDE AND COPPER SULPHATE CATALYST AS AN ADDITIONAL FEED AND ITS EFFECT ON INTESTOLOGICAL HISTOLOGY OF SENTUL CHICKEN

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Abstract

The study was carried out experimentally, the first identified noni fruit extract on yield, and bacterial inhibition and the second was the application of noni fruit extract supplemented with Cu and Zn on intestinal histology. The first stage used a nested complete randomized design (CRD) and the second stage used RAL and was further tested with Duncan Multiple Range Test (DMRT). The livestock used were 100 DOC (Day Old Chicken) unsexing Sentul chickens, reared for 12 weeks. Data were analyzed using SAS JMP Pro version 14 software. The results showed that methanol solvent with a maceration time of 48 hours was the best treatment for producing yield, and inhibition of E. coli and S. aureus bacteria. The best treatment was the administration of 250 mg/kg of noni fruit extract supplemented with Cu and Zn. The conclusion is that the addition of noni fruit extract with Cu and Zn supplementation in the ration up to a level of 250 mg/kg can increase the height, width, and depth of the crypts and it is recommended to use it as a feed additive to replace Antibiotic Growth Promoter.

Key words*: antibacterial, intestinal villi, Noni fruit extract, Sentul chicken, yield.*

INTRODUCTION

Noni fruit is an herbal plant that has the potential to be used as additional feed to replace Antibiotic Growth Promoters (AGP). Noni fruit contains some phytogenic compounds and active substances that function as antibacterial, antioxidant, antihelmintic, and anti-cholesterol. The content of antibacterial compounds in noni fruit can suppress the activity of pathogenic microbes in the small intestine, so that it can increase the efficiency of the feed ingredients utilization. Evaluation of the efficiency of utilization of feed ingredients can be done by measuring the digestibility of animal feed ingredients. The higher the digestibility value of a feed ingredient, the more nutrients that are utilized by livestock. The surface area of the intestinal epithelium, the height of the villi, and the number of villi and microvilli will affect the absorption and digestibility of feed ingredients. The higher and wider the intestinal villi, the more nutrients are absorbed and digested. Anthraquinones are antibacterial and antioxidant compounds contained in noni fruit. Anthraquinones can also increase the absorption of ration protein by affecting the pH of the digestive tract to become more acidic. Noni fruit also contains scopoletin, which is a group of simple coumarin compounds that have antibacterial and immunomodulatory activity. The utilization of noni fruit as an additional feed ingredient for poultry must be processed first, because the active compound content of noni fruit is volatile due to improper treatment. Extraction is the main process that can extract antioxidant and bioactive phenolic compounds from a material. Extraction aims to maximize the number of target compounds withdrawn to obtain optimal biological activity in the livestock body. The resulting extraction results are not only influenced by the extraction technique, but also by the extraction solvent. Various kinds of solvents can be used in the extraction process, ranging from polar, semipolar, non-polar, or water solvents, which are usually adjusted to the solubility properties of each solvent according to the compound to be extracted. Some of the things, that affect the extraction of a material, include the type of solvent used and its concentration, solvent ratio, contact time, temperature, and the size of the solid particles to be extracted, besides that the addition of a catalyst to an extract plays a role in influencing the effectiveness and efficiency of the target compound in the body. Copper sulphate ($CuSO_4$) and zinc oxide (ZnO) are catalysts that can be added to plant extracts. In addition to their role as catalysts, Cu and Zn minerals are also needed in the process of metabolizing food substances in the digestive tract and as metalloenzyme activators. Based on the above background, it is necessary to conduct a study on the utilization of noni fruit extract with ZnO and $CuSO_4$ catalysts as additional feed and its effect on the intestinal histology of Sentul chickens.

MATERIALS AND METHODS

Study area

This study was divided into two stages. Stage 1 was the determination of the solvent and maceration time of noni fruit extract, which produced the greatest yield and inhibition of bacteria. Phase 2 of the study was to determine the best dosage of adding noni fruit extract with ZnO and $CuSO_4$ catalysts into the ration and its effect on the histology of the jejunum of Sentul chickens. The materials used in this study were ripe noni fruit, 96% ethanol, methanol, n-Hexane, chloroform p.a, $CuSO_4$, ZnO, filter paper, methanol p.a, DPPH, ascorbic acid, distilled water, Muller Hinton Agar (MHA), amoxicillin, physiological NaCl, 70% alcohol, sulfuric acid, $BaCl_2$, blank disk, *Escherichia coli* and *Staphylococcus aureus* bacterial cultures, Sentul chicken and Bouin solution. Research Procedure Phase 1 (Sogandi & Rabima, 2019 with modifications) Noni fruit powder is dissolved with several solutions including ethanol, methanol, chloroform, and n-hexane with a ratio of 1: 3. The noni fruit solution was macerated for 24 and 48 hours. After that, the noni fruit solution was filtered using filter paper and concentrated using a rotary evaporator until it became a solid extract, then analysis was carried out on the inhibition of *E. coli* and *S. aureus* bacteria. Data were analyzed using a nested completely randomized design (CRD). The real effect on the measurement parameters was further tested with the Duncan Multiple Range Test (DMRT). The treatment applied in phase 1 research can be seen in Table 1.

Table 1. Noni fruit extraction treatment

Treatment	W1 (24 Hours)	W2 (48 Hours)
P0 (Ethanol)	P0W1	P0W2
P1 (Methanol)	P1W1	P1W2
P2 (Chloroform)	P2W1	P2W2
P3 (*n*-Hexana)	P3W1	P3W2

Phase II Research Procedures

The experimental design used in this phase 2 study was a Completely Randomized Design (CRD). A total of 100 DOC Sentul chickens (unsexing) were grouped into 5 feed treatment groups. The feed treatment applied to each experiment was repeated 4 times. Maintenance is carried out for 12 weeks. Treatment feed was given to livestock in the 2nd to 12th week. The feed treatment applied is P0 = 100% Basal ration (RB), P1 = 100% RB + 50 mg/kg Zinc bacitracin, P2 = 100% RB + 125 mg/kg Noni extract with ZnO and $CuSO_4$ (EBMM), P3 = 100% RB + 250 mg/kg EBMm, P4 = 100% RB + 375 mg/kg EBMm. After 12 weeks of rearing, the chickens were slaughtered and prepared for histological analysis of the jejunum (Iji et al., 2011). The Sentul chicken was dissected from the chest to the neck and its digestive tract was removed. The intestine is cut in the duodenum to the ileocaecocolic junction and the large intestine, then cleaned of fat and mesentery using NaCl. The posterior jejunum of the small intestine is cut 2 cm long and put into a bottle to be fixed in a Bouin solution, then closed tightly and stored for 24-48 hours. After that, remove the small intestine sample from the bottle and put it in another container to be dehydrated with alcohol with graded concentrations (70%, 80%, 90%, and 100%). After that, the sample was cleaned using xylol and blocked into paraffin. Then the sample in the paraffin block container is soaked in cold water to dry the paraffin wax. Furthermore, the intestinal sample was removed and cut with a microtome 5 μm thick and transferred to a warm water bath. Samples in a floating state are taken and attached to the object glass. Then, the sample on the glass object was dried at 37°C, after drying it was painted by immersing the glass object in a container containing haematoxylin solvent for 10 minutes, then rinsed with running water and dipped in alcohol, then dipped in eosin-solvent for 10 minutes. Take measurements using a microscope with the help of a computer on intestinal histology preparations that are ready in glass objects.

RESULTS AND DISCUSSIONS

Yield and Inhibitory Power of E. coli and S. aureus Bacteria

Noni (*Morinda citrifolia* L.) is a tropical plant that has been used as a food ingredient, spice, and traditional medicine in Southeast Asia for more than 2000 years (Motshakeri & Ghazali, 2015; Yang et al., 2010). Noni fruit contains active components of phenolic compounds, especially coumarins, flavonoids, and iridoid compounds which are useful as antioxidants, antibacterial and antiallergic (Saraphanchotiwitthaya & Sripalakit, 2015). In its ability as an antibacterial, noni fruit can inhibit the growth of several types of bacteria, both Gram-negative and Gram-positive (Azizah & Widjastuti, 2021). Based on the results of statistical analysis, the type of solvent, different solvents, and maceration time had a significant effect (P<0.05) on the yield and inhibition of the bacteria produced. The average yield and inhibition of gram-positive and negative bacteria for each treatment can be seen in Table 2 below.

Based on the results of statistical analysis, the difference in the type of solvent had a significant effect (P<0.05) on the percentage of extract yield, but maceration time had no significant effect (P>0.05). Duncan's test showed that the yields produced from polar solvents (ethanol and methanol) were not significantly different from each other, but significantly different from non-polar solvents (chloroform and n-hexane). The amount of yield produced depends on the solubility of the bioactive components of the sample extracted. The solvent will diffuse into components that have the same polarity level so that the solvent which has the same polarity level as the extracted bioactive compounds will produce a greater yield. The order of the largest yield based on treatment was P1, P2, P3, and P4 namely 21.50%, 20.72%, 4.97%, and 3.81%. Based on this, it is suspected that the active compounds contained in noni fruit mostly consist of polar compounds. The large value of the yield of methanol extract is caused by its polar nature, so it can dissolve almost all organic compounds present in the sample, both polar and non-polar compounds. Research Jacobeb et al (2011) on api-api leaves (*Avicenia marina*) showed that more extract content was obtained using methanol compared to other solvents. The results of this yield are in line with the inhibition of *E. coli* and *S. aureus* bacteria, the average noni fruit extract extracted with polar solvents is higher than non-polar solvents.

Table 2. Average yield and inhibition of noni fruit extract bacteria

Variabel		Treatments							
		P1		P2		P3		P4	
		W1	W2	W1	W2	W1	W2	W1	W2
Yield	Average P(W) (%)	21.65± 2.98	21.35± 3.32	21.67± 10.81	19.77± 5.28	4.91± 1.00	5.03± 1.33	4.31± 2.38	3.3± 1.34
	Average P (%)	21.50 ± 2.83^{b}		20.72 ± 7.68^{b}		4.97 ± 1.05^{a}		3.81 ± 1.81^{a}	
Inhibition *E. coli*	Average P(W) (mm)	6.70 ± 0.37^{a}	12.17 ± 0.29^{d}	6.56 ± 0.64^{a}	12.96 ± 0.59^{d}	7.91 ± 0.36^{bc}	8.65 ± 0.21^{c}	7.62 ± 0.39^{b}	6.73 ± 0.20^{a}
	Average P (mm)	9.48±2.96		9.76±3.55		8.28±0.48		7.18±0.56	
Inhibition *S. aureus*	Average P(W) (mm)	15.47± 0.46	15.50± 1.25	14.27± 0.95	16.35± 1.43	11.29 ±1.05	11.63± 0.06	7.93 ±0.08	7.51 ±0.68
	Average P (mm)	15.48 ± 0.84^{c}		15.31 ± 1.57^{c}		11.46 ± 0.69^{b}		7.75 ± 0.75^{a}	

Different superscripts on the same line show significant differences (P<0.05)

P1 = Ethanol, P2 = Methanol, P3 = Chloroform, P4 = n-Hexane, W1 = 24 hours, W2 =48 hours

Table 3. Average histology of the small intestine of Sentul chickens fed AGP and noni fruit extract at various levels

Variable	Treatment				
	P0	P1	P2	P3	P4
Villi Height (µm)	692.479±29.980^{a}	810.51±35.252^{c}	735.408±86.065^{b}	833.863±13.731^{d}	747.555±42.843^{b}
Villi Width (µm)	133.563± 3.756^{c}	109.437±4.692^{a}	109.358 ± 4.189^{a}	141.995 ± 6.707^{d}	125.998 ± 1.031^{b}
Crypte Depth (µm)	51.947 ± 1.449^{b}	45.633 ± 2.334^{a}	57.031 ± 1.805^{b}	66.295 ± 3.105^{c}	57.328 ± 8.537^{b}

Different superscripts on the same line show significant differences (P<0.05)
P0 = basal diet, P1 = basal diet + 50 mg/kg zinc bacitracin, P2 = basal diet + 125 mg/kg noni fruit extract, P3 = basal diet + 250 mg/kg noni fruit extract, P4 = basal diet + 375 mg/kg noni fruit extract

Based on the results of statistical analysis, the time treatment and the type of solvent used for noni fruit extraction had a significant effect on the inhibition of *E. coli* and *S. aureus* bacteria. Time nested in the solvent showed a significant difference (P<0.05) in the inhibition of *E. coli* bacteria. Polar solvents (ethanol and methanol) with a maceration time of 48 hours have a greater inhibition of *E. coli* bacterial activity than the same solvents with a maceration time of 24 hours, as well as other treatments. The inhibition power of *E. coli* bacteria in noni fruit extract was P2W2, P1W2, P4W2, P3W2, P4W1, and P4W2 respectively with the largest inhibition power of *E. coli* 12.96 mm. Longer extraction times can extract greater antibacterial compounds compared to extracts with shorter maceration times. Extracts with a maceration duration of 48 hours had an average inhibition of *E. coli* bacteria that was greater than extracts macerated for 24 hours. The use of the type of solvent alone had a significant effect (P<0.05) on the inhibition of *S. aureus* bacteria. This shows that both solvent and maceration time have a significant effect on the inhibition of Gram-negative and Gram-positive bacteria. The ability of noni fruit extract to inhibit these bacteria is due to the presence of iridoids (deacetylasperulosidic and asperulosidic acids) (Deng et al., 2011), secondary metabolites such as phenols, steroids, terpenoids, alkaloids, tannins, flavonoids, saponins, glycosides, reducing sugars, and acid compounds (Anugweje, 2015). Wall et al. (2015) reported that organic acids in noni fruit juice include acetic, ascorbic, butyric, citric, dehydroascorbic, galacturonic, malonic, succinic, shikimic, and tartaric acids.

The formation of the inhibition zone area for gram-positive bacteria is generally larger than the inhibition zone formed for Gram-negative bacteria. Noni fruit extract was able to inhibit the activity of *S. aureus* bacteria by 16.35 mm, while the inhibition of *E. coli* bacteria was 12.96 mm. This is due to the characteristics of each bacteria that are different from one another. According to Sudewi & Widya (2016), the difference in the bacterial inhibition of noni fruit extract occurs due to the structure of the bacterial cell wall which affects the sensitivity of the bacteria. Gram-positive bacteria tend to be more sensitive to antibacterial compounds, making it easier for antibacterial compounds to enter gram-positive bacterial cells. The structure of the cell wall of Gram-positive bacteria is simpler than the structure of the cell wall of Gram-negative bacteria, so the penetration of antibacterial compounds in Gram-positive bacteria is easier than that of Gram-negative bacteria.

Based on the research results on the yield value and inhibition of *E. coli* and *S. aureus* bacteria produced, the ethanol and methanol extracts of noni fruit with a maceration time of 48 hours had a higher average than other treatments. The ethanol extract of noni fruit with a maceration duration of 48 hours had a yield of 21.35%, the inhibition of *E. coli* and *S. aureus* bacteria were 12.17 mm and 15.50 mm respectively, while the methanol extract of noni fruit was 19.77% respectively, 12.96 mm and 16.35 mm. The results of these two treatments were not significantly different in each measurement parameter, meaning that both have the same potential to be applied in the second stage of the study.

Histology of intestines

The digestive tract has an important function in absorbing nutrients. In animal production systems, a healthy gut is required to achieve the best performance results, and the concept of gut health can be summarized as a state of gut homeostasis. The results of observations of the histology of the Sentul chicken's jejunum can be seen in Table 3.

Based on Table 3, it is known that the supplementary feeding of noni fruit extract with the supplementation of Cu and Zn minerals had a significant effect ($P<0.05$) on the height of the small intestinal villi of Sentul chickens. P0 is significantly different from P1, P2, P3, and P4, but P2 and P4 are not significantly different from each other. P3 has a higher average villous height compared to P0, P1, P2, and P4. The order of the largest intestinal villi height was P3 = 833.863 µm, P1 = 810.51 µm, P4 = 747.555 µm, P2 = 735.408 µm and P0 = 692.479 µm. The average height of small intestinal villi of Sentul chickens given a control diet and with the addition of AGP was smaller than the average height of intestinal villi given an additional 250 mg/kg of fruit extract with Cu and Zn supplementation. This shows that the level of addition of 250 mg/kg of noni fruit extract in the ration is the optimal level in producing the best intestinal villi height. Singh et al. (2012) stated that *Morinda citrifolia* has bioactive compounds that function as antimicrobials that can suppress *E. coli* in the small intestine, so the antimicrobial activity of noni fruit extract added to the ration may be the cause of the increase in villi height compared to controls. Satimah et al. (2019) added that increasing the length of the small intestinal villi causes a wider surface area for absorption, so that nutrient absorption becomes more optimal.

The results showed that the provision of additional feed in the ration had a significant effect ($P<0.05$) on the villi width of the Sentul chicken's small intestine. P0 is significantly different from P1, P2, P3, and P4, but P1 and P2 are not significantly different. P3 had the largest average villi width of 141.995 µm, followed by P0 = 133.563 µm, P4 = 125.998 µm, P1 = 109.437 µm and P2 = 109.358 µm. The addition of 250 mg/kg of noni fruit extract with Cu and Zn supplementation in the ration was the best treatment in producing the largest width of the villi compared to the positive and negative control and other treatments, this is due to the presence of an active compound in noni fruit, namely xeronine, which can help dilate the small intestine and make the intestinal villi widen. The higher and wider the intestinal villi will further expand the surface of the intestinal villi, so that the absorption of nutrients will also increase. emphasized by Asmawati (2014) that the wider the villi, the more nutrients will be absorbed, so that it can have an impact on organ growth, and the carcass will increase. In addition, the anthraquinone active substances contained in noni fruit can stimulate the growth process of the intestinal villi and affect the absorption of feed and digestive activity in the intestine (Widjastuti et al., 2023).

Based on the results of the study in Table 3, the administration of noni fruit extract into the ration had a significant effect ($P <0.05$) on the depth of the crypts. P0 has no significant effect on P2 and P4 but has no significant effect on P1 and P3. The highest mean jejunum crypt depth was in treatment P3, which was 66,295 µm and the lowest was in treatment P1, which was 45,633 µm. The depth of the crypts of the small intestine of Sentul chickens fed with feed additive treatment of 250 mg/kg of noni fruit extract with Cu and Zn supplementation was much greater than that of the treatment without administration of noni fruit extract and with the provision of AGP feed additives, this was due to the dose of 250 mg/kg Noni fruit extract is the most optimal dose in impacting the growth of small intestinal crypts, in contrast to the smaller intestinal crypt depth when given noni fruit extract at doses of 125 mg/kg and 375 mg/kg in Sentul chicken rations. The crypt depth of the small intestine of Sentul chickens, which was higher compared to other treatments, was in line with the results obtained from observations of villi height, villi width, and villi surface area which was larger than the other treatments. This indicates that the treatment of noni fruit extract feed additive with Cu and Zn supplementation at a dose of 250 mg/kg is the optimal dose for administration in the ration.

Increasing the height, width, surface area of the villi, and depth of the crypts of the small intestine will affect the increase in nutrient digestibility of feed ingredients. Nutrient absorption can be affected by the surface area of the intestinal epithelium, the number of folds in it, the height of the villi, and the number of villi and microvilli which expand the area of absorption (Ruttanavut et al., 2009). Awad et al. (2009) stated that an increase in the height and width of the villi in the chicken intestine is closely related to an increase in digestive function and absorption function due to the wider area of absorption of nutrients throughout the body's tissues. One of the parameters that can be used for growth performance is the length and morphological structure of the intestine (Fitasari, 2012).

CONCLUSIONS

The best solvent and maceration time of noni fruit in producing yield, and inhibition of *E. coli* and *S. aureus* bacteria is a methanol solvent with a long maceration time of 48 hours. The optimal dose of adding noni fruit extract with Cu and Zn supplementation in producing villi height, villi width, and crypt depth is 250 mg/kg

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COMPARATIVE STUDY ON THE DYNAMICS OF COWS, MILK PRODUCTION AND DAIRY PRODUCTS

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Abstract

The aim of this study is to achieve a comparative analysis of the cow livestock number, production of milk and of main dairy products in Romania and European Union. The work is an extensive bibliographic documentation with the application of statistical tests for the period 2017-2021. At European Union level the herd of dairy cows is in decreasing with 5.63% and in Romania with 7.95%, from 1175.4 thousand heads in 2017 to 1081.9 thousand heads in 2021. The largest amount of raw milk available on the farm in 2021 belongs to Germany which records 32531.56 thousand tons, approx. 21% of the total quantity at EU level. In 2021 compared with 2017, the Europeans consumed with 3.91% less fresh dairy products and with approx. 5% more cheese. In the future, will be exploit more productive animals, making cow rearing a part of solutions in food crisis.

Key words*: dairy, EU, herd, milk, Romania.*

INTRODUCTION

The future development of the animal husbandry sector, mainly that of raising of dairy cows, is subject to the objectives of the European Union which aim is to reduce greenhouse gas emissions (GES) by at least 55% until 2030 compared to those in 1990 level and achieve climate neutrality until the year 2050.
Globally, 26% of total greenhouse gas (GES) emissions are generated by food production, most of which comes from agriculture. At the EU level, it is considered that 10.3% of the total emissions come from agriculture, of which the contribution of the livestock sector is 70% www.op.europa.eu). From studies, animal CH_4 emissions from enteric fermentation represents about 17% of global CH_4 emissions (Pulina et al., 2022). The cause is represented by the process of anaerobic fermentation of fodder in the rumen, as a result of which methane is produced, which is a source of pollution for the environment (Knapp et al., 2014).
As a branch of agriculture, animal breeding has generated essential income, even at household level, especially in developing countries (Podar & Oroian, 2003; Stoica & Vladu, 2002). Milk, as a product of animal origin, is a food with major biological value, being recommended for human consumption at any age because it is a source of essential amino- acids and micronutrients such as vitamin B12, vitamin D, iodine, calcium, iron and zinc (Acatincăi, 2004; Henchion et al., 2021; Dinescu & Tontsch, 2002; Georgescu & Militaru, 2003). Animal products contribute with 18% at global calories and with 25% at global protein consumption (Barbieri et al., 2022).
It should not be forgotten that livestock plays a positive role not only for population feeding but also in agroecosystems (Capper, 2012; Man et al., 2002). Herds of cows close nutrient cycles at the farm level and support productivity of crops by providing manure (Barbieri et al., 2022).
The world's population continues to grow and that represents a great challenge for the future related to food security, because it must be found solutions to satisfy increasing demand for protein products of animal origin (Peyraud, & MacLeod, 2020). Among farm animals, ruminants have the ability to convert vegetable foders into protein and they are almost the only source of milk for humans (Barbieri et al., 2022).
The largest amount of milk processed to obtain dairy products comes from cows exploited for

this production. At the level of year 2021, the world production of cow's milk was 746.056 million tons, compared to 20.725 million tons of goat milk, respectively 10.504 million tons sheep's milk. This represents 95.98% of the total milk production (fao.org/faostat). At European Union level, the total production of cow's milk was 154.093 million tons, sheep's milk 2.985 million tons and goat's milk 2.5 million tons, which means that cow's milk production represent 96.56% of the total (fao.org/faostat).
Because of importance of milk as food and the fact that milk from cows is the main source of obtaining dairy products, husbandry of dairy cows has and it is a priority in the breeding of other animals for this production (Maciuc, 2006).
In this framework, the paper presents the evolution of the population of cattle, that of total milk production and productions of the main dairy products obtained by processing cow's milk. The study compares data over a period of 5 years (2017-2021), detailed at global, EU and national levels.

MATERIALS AND METHODS

In order to achieve the goal proposed by this paper, the following indicators were used: the number of cows, the density of cattle in different european countries (heads/100 ha), the total milk production (raw milk), the amount of drinking milk and the productions of the main dairy products such as butter and cream, cheese and yogurt. The statistical estimators that characterize a normal distribution, such as the mean were calculated and on the other hand, the dispersion indices represented by the variance and the standard deviation. Statistics are written with latin letters: arithmetic mean ($\overline{X}$), variance (s^2), standard deviation (s) and parameters with greek letters: theoretical mean (μ), variance ($ó^2$) and standard deviation (ó) (Cucu, I. et al., 2004)
The data that have been processed are publicly provided by institutions such as FAO, Eurostat and the National Institute of Statistics (Romania) in the case of national data.
It should be mentioned that the data analysis was carried out through the prism of combining and correlating with the observations made directly in the field and with the reporting of the results obtained according of requirements and norms of the European Union (EU).

RESULTS AND DISCUSSIONS

In according to data provided by the FAO, in world the number of cattle has increased year-on-year between 2017 and 2021. Thus, if in the first reference year a herd of 1,477.355 million heads was reported, in 2021 it will reach at 1,529.296 million heads, which represents an increase of 3.39%.
However, in the European Union, the cattle herd decreased, reaching 75,655,220 heads in 2021, compared to 79,602,306 heads in 2017, recording a reduction of 4.96%. The decrease of herds is a consequence of the productive superiority of the animals (with very good genetic value), the technologies applied in intensive breeding systems but also as a result of the objectives set by the EU in the short and medium terms regarding the reduction of GES, the sector livestock being directly targeted because it is considered to have a notable contribution to environmental pollution.

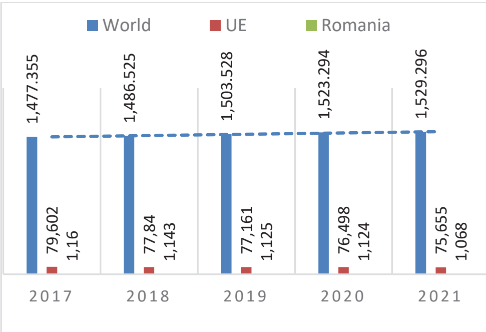

Figure 1. Dynamics of livestock in world, European Union and Romania in the period 2017-2021 (million heads)

Among the member countries of the European Union, France has the largest herd of cattle, but even in this country is a reducing of number of animals by 6.73%, from 18,580 thousand heads in 2017 to 17,330.1 thousand heads in 2021 (www.fao.org). France is followed by Germany, which in 2021 had an effective number of 11,039.7 thousand heads, decreasing by 10.1% if compared to 2017.

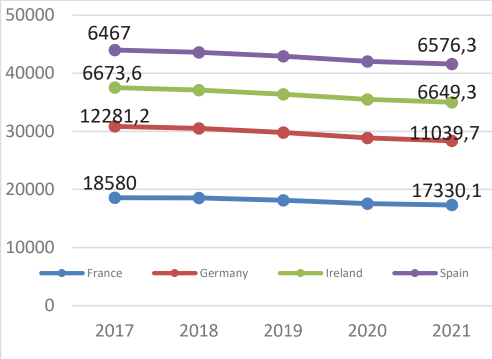

Figure 2. Evolution of livestock in the main EU member countries (thousand heads)

Holland is the EU member country that has the highest density of cattle, in 2021 this was 208.5 heads/100 ha. In second place was Belgium with 177.6 head/100 ha, followed by Ireland with an animal density of 145.3 head/100 ha. Romania ranks 20th in the hierarchy of member countries, with a cattle density of 14.3 heads/100 ha, ahead of Bulgaria and Greece.

Regarding worldwide milk production, this increased in 2021 compared to 2017, as a result of increased demand for animal products caused by population growth. The highest average production during the analyzed period belongs to Asia, which achieved 31.9% of the total global production, followed by Europe with 31.3% and by the American continent whose average production represented 26.8% of the total. In 2017, total milk production worldwide was 685.199 million tons and in 2021 it increased by 8.16%, reaching at 746.057 million tons.

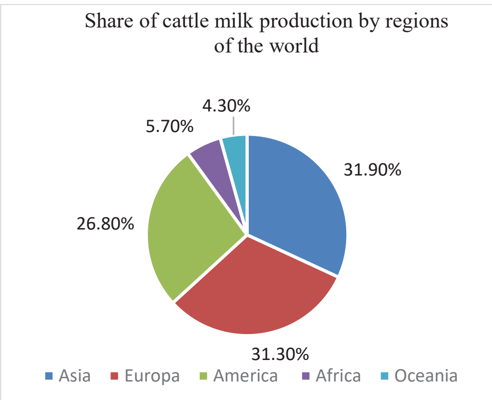

Figure 3. Average global raw cattle milk production

In 2017, Europe achieved a production of 221.095 million tons of milk (32.3% of global production), which placed it in first place in the world ranking, followed by Asia, whose production then represented 30.3% of the total, and America with a percentage of 27.4%.

In 2021, the continent with the biggest milk production is Asia with 248.578 million tons, which represents 33.3% of world production. This increase of production is the answer to the increased demand for dairy products, a large part of the population of the Earth is concentrated in this area of the world. India is the country with the largest milk production 108.3 million tons of raw milk, ranking first both among countries in the region and in the world, the production achieved means 43.57% of Asia's production and 14.5% of the entire world production. Among the countries of the world, India is followed by the USA which produced 102.629 million tons and then by China with a production of 36.827 million tons.

In the European Union, milk production increased year-on-year from 2017 to 2020, from 148.814 million tons to 154.487 million tons. The year 2021 record a slight decrease in production to 154.093 million tons, with 0.25% less than the previous year. The geopolitical conditions on the European continent but also the drought of 2021 led to this reduction of production.

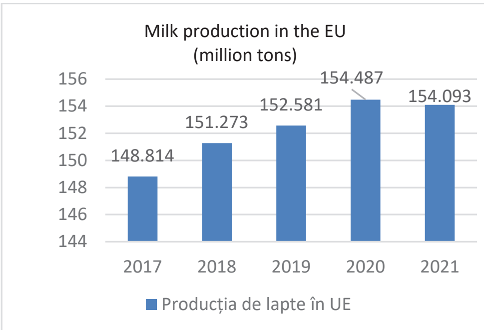

Figure 4. Graphic representation of milk production in the EU in the period 2017-2021 (million tons)

Germany is the country with the highest average milk production among the EU member countries, in the analyzed period. In 2017 it had a production of 32.598 million tons of milk and in 2021 of 32.507 million tons, which represented 21.9% and respectively

21.09% of the production of the European Union.

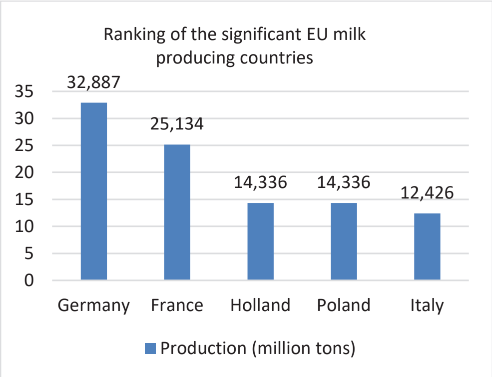

Figure 5. Ranking of the first EU member states according to the total average milk production of the period 2017-2021 (million tons)

Although Germany has a cattle density about 75 heads/100 ha, the productions achieved are the highest among the countries of the union. In the ranking it is followed by France, the Netherlands, Poland and Italy, these countries together achieving 65.1% of the total average EU production of the period, of 152.25 million tons.

Milk production per cow differs betwen EU member states, being the highest in Denmark (10,097 kg) and Estonia (10,020 kg) and the lowest in Bulgaria (3,628 kg) and Romania (3,362 kg) (agrointel.ro).

The genetic base of the animals, the performance of the applied technologies are some of the factors that determine the individual performances of the animals.

By processing milk, plus value is added to the product and dairy production is closely correlated with market demand. Analyzing the statistical data provided by Eurostat, we can conclude that in the EU member countries, production of drinking milk has decreased quantitatively, registering in 2021 compared to 2017 a reduction of 3.22% in Germany, of 3.80% in Spain and of 9.37% in France (ec.europa.eu/eurostat).

On the other hand, the production of cheese obtained from cow's milk (skimmed and whole) increased from year to year. Thus, in 2017 there was a production of 8.789 million kg and 10.690 million kg in 2021, which means an increase of 21.63%.

Poland is one of the member countries where there is an increase for most of dairy productions, being one of the states where raw milk production increased annually during this period. Thus, in 2021 compared to 2017, drinking milk registers a percentage increase of 13.08%, yogurt production is higher by 7.37%, butter by 8.63% and cheese by 9.34%. For cheese production, Poland is overtaken by Spain, where the increase is with 13.96% higher.

In Romania, raising dairy cows is one of the important sectors of agriculture. Compared to the analyzed period, it can be stated that at the national level there is a decrease of 8.22% in the number of milk cows, from a herd of 1,160,136 heads in 2017 to 1,064,758 heads in 2021. If we were to analyze the distribution of the herd in the territory, it is found that most cows are raised in the NE area of the country (Suceava and Botoșani counties) at the opposite pole being the Bucharest-Ilfov area.

The Central region of the country is the only one in which an increase in the herd of milk cows is recorded, the number of cows being higher by 6.91% in 2021 compared to 2017. In the other regions there are decreases in herds, the largest being in the Bucharest-Ilfov region of 50.63% (halving) but also in the regions affected by drought in recent years such as SE and SW Oltenia where the herd reductions are 19.78% and 17.28% respectively.

Table 1. Evolution during 2017-2021 of dairy production in the main producing countries (1000 tons)

Dairy product	The country	Year					Average production of the period (2017-2021)	2021/2017 (%)
		2017	2018	2019	2020	2021		
Drinking milk	Germany	4595.13	4452.07	4597.32	4634.84	4447	4545.27	-3.22
	Spain	3538.05	3292.22	3184.15	3504.5	3403.5	3384.48	-3.80
	France	3299.06	3212.61	3172.7	3157.59	2989.87	3166.36	-9.37
	Italy	2459.03	2469.56	2479.07	2448.89	2488.3	2468.97	+1.19
	Poland	1733.6	1778.55	1892.17	1988.13	1960.38	1870.56	+13.08
Yogurt	Germany	1898.66	1889.56	1864.18	1829.67	1752	1846.81	-7.72
	Spain	1022.4	1021.87	957.12	903.28	981	977.13	-4.05
	France	1449.7	1453.26	1333.01	1381.58	1309.85	1385.48	-9.65
	Italy	325.01	275.98	252.8	278.76	280.4	282.59	-13.73
	Poland	510.16	532.24	538.64	538.47	547.75	533.45	+7.37
Butter	Germany	488.11	474.88	490.65	497.30	461.68	482.52	-5.41
	Spain	51.19	50.99	48.65	49.81	52.25	50.58	+2.07
	France	412.72	417.41	419.22	417.54	410.54	415.48	-0.53
	Italy	91.2	97.48	94.03	92.25	94.15	93.82	+3.23
	Poland	213.72	222.66	224.45	243.39	232.17	227.28	+8.63
Cheese	Germany	2216.55	2245.8	2297.4	2355.12	2360.9	2295.15	+6.51
	Spain	481.12	474.68	442.23	471.84	548.31	483.64	+13.96
	France	1919.57	1907.76	1903.29	1862.10	1865.96	1891.74	-2.76
	Italy	1261.13	1308.03	1327.3	1344.69	1374.23	1323.07	+8.98
	Poland	840.63	855.59	867.95	893.78	919.17	875.42	+9.34

Table 2. Dynamics of livestock at national level by development regions (heads)

Development region	2017	2018	2019	2020	2021	Effective averages (Ef_m) per period	2021/ 2017 (%)	Ef_m region/ Ef_m total country (%)
North-East (NE)	274,851	269,550	257,649	259,788	246,479	261,663.4	- 10.32	23,28
South-East (SE)	125,457	127,428	116,836	107,363	100,636	115,544	-19.78	10.28
South Muntenia	139,572	134,029	131,020	123,309	111,093	127,804.6	-20.4	11.37
South-West (SW) Oltenia	109,075	105,108	105,098	100,084	90,221	101,917.2	-17.28	9.07
West (W)	92,026	91,958	93,771	93,457	89,042	92,050.8	-3.24	8.19
North-West (NW)	207,493	205,100	210,151	215,616	205,319	208,735.8	-1.05	18.57
Center	207,506	206,777	207,807	222,055	222,916	213,412.2	+6.91	18.98
Bucharest - Ilfov	4,156	3,286	2,477	2,582	2,052	2,910.6	-50.63	0.26
Effective per country	1,160,136	1,143,236	1,124,809	1,124,254	1,064,758	1,124,038	-8.22	-

source: www.inss.ro

Table 3. Dynamics of the total production of cow's milk and dairy products obtained in Romania for the period 2017-2021 (tons)

Product	Reference years					Average production of the period (2017-2021)	2021/2017 (%)
	2017	2018	2019	2020	2021		
Total milk production	4,159,637	4,168,975	4,077,401	4,125,065	4,021,539	4,110,523.4	-3.32
Milk consumption	1,115,539	1,088,198	1,046,594	1,080,887	1,097,997	1,085,843	-1.57
Amount of milk for cheese	1,409,003	1,334,948	1,218,237	1,232,144	1,164,466	1,271,760	-17.36
Amount of cheese	214,248	211,410	189,864	188,700	175,635	195,971.4	-18.02
Amount of milk for yogurt	21,705	24,194	21,364	24,805	20,010	22,415.6	-7.81
Amount of milk for butter and cream	152,630	158,446	152,918	148,373	158,510	154,175.4	+3.85
Amount of butter and cream	15,699	18,608	16,835	16,622	16,097	16,772.2	+2.54

From the data made available by the National Institute of Statistics, it appears that in Romania, the total average milk production of the period was 4,110,523 tons of milk, decreasing by 3.32% in 2021 compared to 2017, from 4,159,636 tons (2017) to 4,021,539 tons (2021).

In the analyzed time interval, the highest milk production was that of 2018, of 4,168,975 tons of milk. Percentage, the largest amount of milk was processed to obtain cheeses, respectively 30.94%, then for human consumption 26.42% and then to obtain butter and cream 3.75%. Production of drinking milk recorded the maximum in 2017 of 1,115,539 tons. This production has decreased two years in a row but shows a slight upward trend in 2020 and 2021.

If we refer to cheese production, it has decreased every year, so that from 214,248 tons in 2017 it reached 175,635 tons in 2021, which represents a quantitative reduction of 18%.

Regarding the cumulative production of butter and cream, it can be observed that during the period 2017-2021 the maximum recorded is in 2018 of 18,608 tons, after which the quantities obtained decreased constantly. (www.ins.ro).

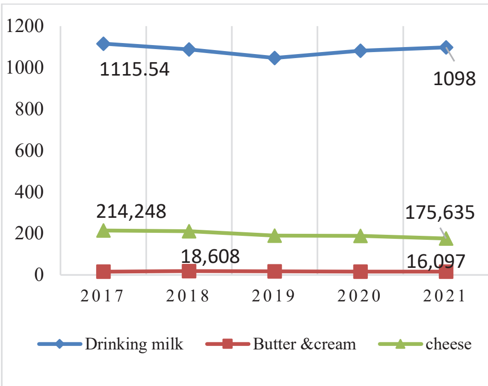

Figure 5. Production evolution of the main dairy products in the period 2017-2021 (thousand tons)

CONCLUSIONS

Animal husbandry and their productions are identified as being among the activities with the greatest impact on the environment.

The objectives that the European Union has proposed to be achieved by 2030 in terms of reducing greenhouse gas emissions and the carbon footprint, aim the measures that directly affect animal husbandry, therefore also the sector of cows breading for milk production. Added to this is the geopolitical solution on the continent.

In this context, dairy herds will continue to decrease numerically. Although overall, statistically, herds gradually decreased from 2017 to 2021, milk production experienced an increase.

At the European level, among dairy products, the largest quantitative increase is the production of cheese obtained from cow's milk, which increased in 2021 by 21.63% compared to 2017 and as a result of the utilization of a larger quantity of skimmed milk on following the reduction in consumption. The demand and production of cheeses has increased due to the preservation of these products for a longer period of time and as a source of very valuable nutrients (proteins).

In the conditions in which the herds of milk cows will follow the descending trend and will decrease numerically in Europe and the European Union while the demand from the population for products of animal origin will increase, it is necessary to find solutions that make cows rearing a sustainable activity in future. Dairy cows farming will have to be integrated as a solution to the food crisis and to support food security and not be seen as an insolvable environmental problem. Some of the measures aim at:

- educating the population regarding the consumption of products of animal origin and reducing food waste;
- ensuring animal welfare which will attract a reduction in administered treatments;
- improving dairy cows for traits that would also have effects on the environment by reducing the carbon footprint, such as: increasing the capacity to digest forage, reducing methane emissions, improving the ability to deal with anti-nutrients in food etc.
- the introduction into the rations of new fodder obtained from crops adapted to the current climatic conditions;
- implementation of technologies related to precision animal husbandry for high performing livestock.

In Romania, a decrease in livestock numbers of 8.22% is observed in 2021 compared to 2017.

This reduction in the number of heads has also attracted a reduction in the total milk production obtained from the animals at the national level.

Cattle density in Romania in 2021 was only 14.4 heads/100 ha, being approximately 14.5 times lower compared to the Netherlands (208.5/100 ha). Given the country's agricultural potential, feed requirements for existing livestock and even increased herds can be met if investment is made in the irrigation system and crop improvement.

For the near future, at the national level, must be found solutions for the maintain and increase of livestock, the application of breeding programs to improve the genetic potential of animals, but also the modernization and expansion of the intensive exploitation system.

It should be considered the financial support and stimulation of dairy farmers considering the challenges they will face in future.

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LIFE CYCLE ASSESSMENT FOR EVALUATING MIXED FARMING SYSTEMS: A REVIEW AND RECOMMENDATIONS

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Abstract

The objective of this work was twofold: i) to characterise the main applications of the life cycle assessment (LCA) for assessing and representing mixed farming systems (MFS), and then ii) to propose a general methodological framework for conducting a comparative LCA of a case study of an MFS versus a specialised system in Romania. For this purpose, the main applications of LCA to MFS have been analysed in all its phases. Overall, the reviewed LCA studies highlighted the potential of MFS to improve environmental sustainability, but scarcity of real data hindered the assessment process. In addition, some studies focused on a single product rather than taking into account all products (crops and livestock) when comparing MFS with specialised ones. This may exclude interactions between farm components in the MFS and therefore may not reflect the overall impact of these systems. Therefore, an LCA based on a farm-level approach is recommended to provide a fairer comparison of MFS versus specialised systems.

Key words: *environmental sustainability, farm-level approach, interactions, mixed farming.*

INTRODUCTION

Reconnecting crops and livestock at the farm and regional level would reduce the ecological footprint, close nutrient cycles, restore ecosystem functions, improve soil health, and increase resource use efficiency.
The positive effects of mixed farming systems (MFS) are mainly proven at the theoretical level (Veysset et al., 2014; Marton et al., 2016), but additional information and knowledge at the practical level are needed regarding their impacts (e.g., pest and disease control, GHGs, biodiversity, etc.) (Shut et al., 2021). MFS are complex multifunctional systems with multiple outputs and different interactions and synergies between farm components. Therefore, structured and specific methodological assessment frameworks are needed to deal with this complexity.
Life Cycle Assessment (LCA) is a standardized method (ISO, 2006a; 2006b) for assessing the environmental impact derived from the life cycle (LC) of products, services and systems.
Despite the growing interest in crop-livestock reintegration as a possible alternative to mitigate the negative effects of agricultural specialization, the literature on LCA of MFS remains scarce compared to other agricultural systems. Modelling the complexity of these systems with LCA is challenging.
In this context, this paper analyses the most relevant applications of LCA to MFS in order to provide an overview of the main characteristics of LCA to represent MFS and to derive a general methodological framework for conducting a comparative LCA of a case study of an MFS versus a specialised system in Romania.

MATERIALS AND METHODS

In order to provide an overview of the main characteristics of LCA to represent MFS, a review of the related scientific literature was conducted, taking into account: (i) recent environmental LCA studies on the MFS and; (ii) the comparison of different steps and components of LCA.
In this review, 8 relevant LCA studies on MFS were identified. These studies have been published between 2012 and 2020. The respective studies have been characterized and

analysed in all the LCA phases (from the definition of the objective and scope to the interpretation of the results) (Figure 1).

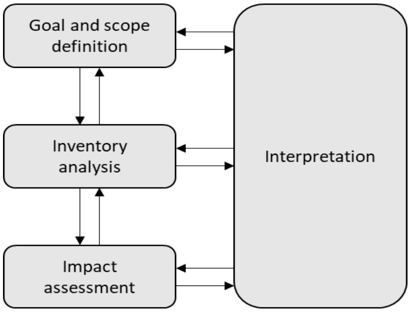

Figure 1. Phases of the common framework for LCA (ISO, 2006a; 2006b)

This review identifies the main components for the design of a methodological framework to conduct a fairer comparative LCA between a case study of MFS and a specialised system scenario in the Alexandria region of Romania.

RESULTS AND DISCUSSIONS

Table 1 shows the main features of the analysed LCA applications to MFS. The LCA applied to MFS was based on the common methodological framework for LCA proposed by ISO 14040 and ISO 14044 (ISO, 2006a, 2006b), which is structured in 4 steps (Figure 1): Goal and scope definition, life cycle inventory (LCI) analysis, impact assessment and interpretation.

The main goals of the analysed LCA applications to MFS were:

- Compare the environmental performance of MFS against other systems (Veysset et al., 2014; Marton et al., 2016; Parajuli et al., 2018; Costa et al, 2018), and alternative MFS scenarios (Vogel et al., 2020);
- Compare the environmental impacts of the different components of MFS (livestock, crops, etc.) (Eady et al., 2012; Parajuli et al., 2018; Paramesh et al., 2019) and;
- Assess the effect of climate change on the environmental impacts of MFS (Tendall et al., 2015).

The scope of these studies was limited to the first stage of the food supply chain i.e., from 'cradle to the farm gate'. In the case of MFS, the farm is mainly divided into two main components: livestock production and crops, which have interactions between them.

Table 1. Main features of the LCA on mixed farming systems

Reference	Scope	FUs	Data sources	Impact categories
Eady et al. (2012)	Cradle to farm gate	- 1 t of grain; - 1 kg of greasy wool; - 1 animal	Farm documents; Literature and agricultural models; Ecoinvent 2.0 unit processes; Australian Unit Process LCI; LCA Food DK Library	GWP
Veysset et al. (2014)	Cradle to farm gate	1 kglw; UAA ha	Field survey (commercial farm data); Literature	GHG (GWP); NRE (with LCA); ANB
Tendall et al. (2015)	Cradle to farm gate	MJ dig. en. for humans	SALCA database; Ecoinvent	NRE; GWP; TOF; AP; FE; MWE; TER; AEP; TEP; HTP; LUC; ABL; RTB
Marton et al. (2016)	Cradle to farm gate	- 1 Kg FPCM; - Basket of products: 1 kg FPCM + CPLA	Swiss FADN; Literature; Experts; Ecoinvent v2.2	nrCED; GWP; aqEN; terrET; K use; P use
Parajuli et al. (2018)	Cradle to farm gate	Basket of products: 1 kgLW-SCC + 1 kgLW-Pigs"	Literature; Country statistcis; Ecoinvent v3	GWP; EP; NRE; PFWTox
Costa et al. (2018)	Cradle to farm gate	Composite FU (technological reference unit, TRU)	On the farm from the farm manager; Interviews; Official publications; Technical reports; Ecoinvent; Boustead Model 5.1	TRU; ARD; CC; PEC; AP; POC; FE; MWE; WS; FEC; LU; BI; SHI
Paramesh et al. (2019)	Cradle to farm gate	Basket of products: Total harvested weight produced at farm gate	Experimental site; Ecoinvent v3	GWP; NRE
Vogel et al. (2020)	Cradle to farm gate	1 kglw of beef cattle for fattening; 1 kg of grain (13% moisture)	Experimental field; Secondary data; Literature; Ecoinvent® v.3.01	GWP; AP; EP; AD

ABL = Potential aquatic biodiversity loss; AD = Abiotic depletion; AEP = Aquatic ecotoxicity potential; ANB= Apparent nitrogen balance; AP = Acidification potential; aqEN = Aquatic eutrophication N; ARD = Abiotic resource depletion; BI = Biodiversity indicators; CC = Climate change; EP = Eutrophication Potential; FU = Functional unit; FE= Freshwater eutrophication; FEC = Freshwater ecotoxicity; GHG = Greenhouse gas; GWP = Global warming potential; HTTP = Human toxicity potential; K = Potassium; kg FPCM= kg fat and protein corrected milk; kgLW = kg liveweight; LU = Land use; MJ dig. en. = Megajoules digestible energy for humans; MWE= Marine eutrophication; nrCED = Cumulative energy demand from fossil and nuclear sources; NRE = Non-renewable energy; P = Phosphor; PEC = Primary energy consumption; PFWTox = Potential Freshwater Ecotoxicity; POC = Photochemical ozone creation; RTB = Reduction of potential terrestrial biodiversity; SCC = Suckler cow calves; SHI = Soil health indicators; TEP = Terrestrial ecotoxicity potential; TER = Terrestrial eutrophication; terrET = Terrestrial ecotoxicity; TOF = Tropospheric ozone formation potential; TRU = Technological reference unit; UAA = Utilized agricultural area; WS = Water scarcity.

Thus, results may vary depending on the LCA approach followed (considering one product "product level" or integrating all products from all activities "farm level") and the way processes are attributed in MFS, especially when comparing these systems to specialized ones (Marton et al., 2016). The main benefits and interactions were found in Eady et al. (2012) and Parajuli et al. (2018). These interactions include the use of livestock manure as fertilizer on field crops and the use of the latter for animal feed (crop stubble, grazing). Eady et al. (2012) included benefits related to minimizing weed control and additional nitrogen (N) deposition for crops through sheep grazing, as well as N fixation by the legume in favour of the next non-legume crop in the rotation and the agronomic benefits of the "break crop" (excluding additional N) that increase cereal yields.

Given the importance of the magnitude of the reference flows, these authors argued the need to model farming systems in a way that recognizes the benefits transmitted between farming activities.

Different functional units (FUs) were used in the analysed LCA applications to MFS. Some are based on the farm approach "basket of products" such as (Marton et al., 2016; Parajuli et al., 2018; Paramesh et al., 2019) and others on the product approach considering one farm product (Veysset et al., 2014). The "basket of products" is a composite FU derived from the "farm approach", which considers all products generated by the farm. This approach is more practical than the "product approach" when assessing MFS, as it allows the whole farm to be considered, instead of focusing on one product of the farm. The product approach leads to limiting the identification of optimization opportunities (Marton et al., 2016). The FU "MJ dig. en." used in Tendall et al. (2015), can combine the dual objective of minimizing environmental impacts per area while maximizing agricultural production per area. In this study, the authors revealed that if "ha × y" were to be used as the FU, the trends observed for global warming potential (GWP) would be reversed. The use of combined FU reflecting other agricultural functions and food qualities, such as nutritional value could provide a more balanced assessment (Tendall et al., 2015). The MFS have multiple functions and can reduce the use of synthetic chemical inputs (fertilizers and pesticides) due to the different interactions in the farm (nutrient recycling, animal grazing, etc).

The MFS are multifunctional systems with multiple products. Dealing with multiple functions and products makes the selection of the FU even more complex for these systems. In any case, the choice of FU depends on the objectives of the LC study and the research typology, and may differ at the discretion of the practitioners (De Luca et al., 2018; Espadas-Aldana et al., 2019).

When applied to MFS, LCA faces the issue of how to adequately model the input-output of different activities involving multiple products and co-products as well as complex mutual interactions. Thus, selecting an appropriate method for allocating inputs to outputs is crucial. Several authors, including Eady et al. (2012) and Marton et al. (2016) showed that different allocation methods could affect the results.

Different co-product handling methods were used in the selected studies. Of these, the studies of Eady et al. (2012) and Marton et al. (2016) stand out, since they included the comparison of the results achieved with these different methods. The authors started the process, following ISO recommendations by dividing the farm into sub-processes. The allocation methods are based on two approaches: (i) attributional (economic and physical allocations) and; (ii) consequential (system expansion). In the literature, it is widely recognized that a consequential approach is more suitable for studying changes in production, while an attributional approach is more appropriate for describing a product. In the case of MFS, Marton et al. (2016) confirmed that system expansion (SE) was suitable to cope with the complexity of MFS, especially when comparing these systems with specialised ones. The SE is a "consequential" approach allowing environmental impacts to be attributed to the main product by modelling co-products as an avoided substitute product (avoided burden). However, SE makes the assessment process more complex, as it requires the collection of more data on substitutes, which are also derived

from multifunctional systems, leading to circular reasoning (Wilfart et al., 2021).
For the other studies, the modelling process was reported in less detail using different methods depending on the objective such as, consequential (Parajuli et al., 2018); attributional for LCI and composite FU to avoid allocation of environmental and social burdens (Costa et al., 2018); mass-based allocation (Paramesh et al., 2019) and economic allocation (Vogel et al., 2020). Overall, important results and conclusions were obtained when investigating and using crop-handling methods in the LCA of MFS, however, more detail and transparency is needed on how these complex systems are modelled.
After setting system boundaries, the second step consists of collecting, quantifying and organizing the necessary data for the different elementary flows of materials, energy and emissions to build up the Life Cycle Inventory (LCI).

A representative LCI of the agro-system is needed to draw valuable conclusions for the decision-making process, which sometimes is not the case, as several LCA studies have pointed out (Nemecek and Erzinger, 2005; Renaud-Gentié et al., 2014, among others). In most of the analyzed LC studies (Table 1), the foreground data were related to statistical data and, therefore reliability and representativeness were not sufficiently taken into account. In addition, direct feedback from farmers and farm technicians is often not included, which is an important aspect in assessing the real environmental impacts of such a complex system as MFS. One notable study (Costa et al., 2018) included interviews with farmers and consultants. Stakeholder participation and farmer involvement is crucial to build a representative LCI model of the system under study and to understand farmers' choices and strategies (Pradeleix et al., 2022) and build useful decision support systems. Regarding background data, the most used LC database is Ecoinvent (Table 1). In general, this LC database and others more specific to the agri-food sector, such as Agribalyse, Agrifootprint, and Food LCA-DK, are based on data from specific times and sites, and should be used accounting for representativeness limitations. Therefore, investigation of site-specific data by LC practitioners is recommended to accurately model consumptions and emissions (Röös et al., 2010; Bellon-Maurel et al., 2014, among others).

Regarding impact categories, the most studied are GWP in kg CO_2eq and non-renewable energy use/demand/consumption (NRE) in MJ eq. Water scarcity was calculated in only one study (Costa et al., 2018). Biodiversity-related impacts were found only in two studies (Tendall et al., 2015; Costa et al., 2018) through the use of the SALCA and AgBalanceTM tools, respectively, while those related to soil health and quality were only included in Costa et al. (2018). Indicators related to biodiversity and soil quality are relevant in the context of MFS but are missing from agricultural LCAs in general (Notarnicola et al., 2017; van der Werf et al., 2020). Impacts related to nutrient use and balances, which are also of particular importance for MFS due to their recycling potential. The latter were only assessed in (Veysset et al., 2014) for N, in Marton et al. (2016) for phosphor (P) and Potassium (K) use and in Costa et al. (2019) for the three elements (N, P, K). Not including these aspects is likely to provide less information in the results when comparing the performance of these systems with others. Thus, more attention should be paid to these issues in future studies through the possible establishment of a common evaluation framework for the selection of relevant impact categories regarding MFS.

With respect to results, the comparison of the performance of MFS with specialized systems from a LCA perspective was reported in two studies (Veysset et al., 2014; Marton et al., 2016) (Table 2). These two studies showed contrasting results in terms of environmental impacts. These differences are mainly due to the different types of management in the evaluated farms and the different methodologies used. According to Veysset et al. (2014), the underperformance of MFS is mainly related to the independent management of MFS production units (livestock and crops) by farmers, which leads to higher input use and a low level of interactions between farm components. This, not only decreased nutrient recycling use and environmental performance, but economic outcomes. In addition, Veyset et al. (2014) focused on livestock products and did not include crop products in the comparison between MFS and specialized farms.

Table 2. Systems studied and main results in analysed LCA applications to MFS

Reference	Systems	Main hotspots/phases	Comparison of systems
Eady et al. (2012)	MFS	Stud rams Lupins	–
Veysset et al. (2014)	MFS and SS	Fertilisers Fuel	- Lower impact: SS - Higher impact: MFS
Tendall et al. (2015)	MFS	Pesticides Livestock-related fluxes for herd replenishment and feed inputs	–
Marton et al. (2016)	MFS and SS	Manure management Methane emission	- Lower impact: MFS - Higher impact: SS
Parajuli et al. (2018)	2 MFS: MFSGB and MFSWB	N_2O emission (fertilisers); Diesel consumption; Methane emission	- Lower impact: MFSGB - Higher impact: MFSWB
Costa et al. (2018)	2 MFS and CS	Livestock emissions Fertilisers; Zinc minerals in cattle feed	- Lower impact: MFS - Higher impact: CS
Paramesh et al. (2019)	MFS	Enteric methane emissions; Diesel consumptions; N_2O emissions (chemical fertilisers)	–
Vogel et al. (2020)	MFS	Methane emissions; Manure; fertilisers	–

MFS = Mixed farming system; SS = Specialised system; CS = Conventional system; MFSGB = MFS with a green biorefinery; MFSWB = MFS without a green biorefinery

In contrast, Marton et al. (2016) followed a "farm approach" by considering all farm products, including the interactions between different farm activities in MFS. Summarizing, taking into account the interactions and benefits (nutrient recycling, animal grazing, etc.) shared between farm activities in MFS, at the level of farmer strategy (practical level) and at the theoretical level, could affect the environmental and economic results at both levels.

In general, the rest of the studies confirmed that good MFS management, taking advantage of and expanding the interactions and synergies between farm activities, could mitigate environmental impacts.

General LCA methodological framework for the case study. The object of the study is a mixed crop-livestock farm in the region of Alexandria in Romania. The livestock farm keeps an average of 200 livestock units (LUs). The LUs include 160 heads of dairy cows and 40 heads of young stock. The crops cultivated in the agricultural area are maize, wheat and barley. Manure resulting from livestock activity is used as fertiliser for the crops, which in turn provides feed for the livestock activity. The animals do not leave the dairy cow farm and do not go to pastures at any time of the year. The goal of the study is to evaluate and compare the environmental impact of this system to a linear scenario (system with disconnected livestock and crops). Figure 2 shows the general diagram and the LCA boundaries for the studied MFS. The scope of the study is "cradle to farm gate", including the crop-livestock production chain from the extraction and use of raw materials and energy (electricity, fuel, water, fertilizers, pesticides, cleaning and medicines products, etc.) to the farm gate (Figure 2).

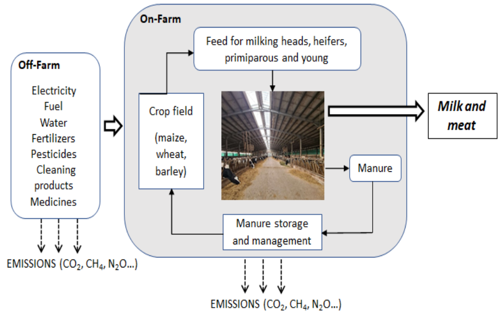

Figure 2. General flow diagram for the case study of MFS

Table 3 presents the main features of the LCA methodological framework for the case study. The main elements of the methodological framework have been derived from the recommendations and findings of the above literature review.

Table 3. Main features of the LCA methodological framework for the case study of MFS

Goal	Scope	FU	Data sources	Co-product handling method
Evaluate and compare the environmenta l impact of an MFS to a SS	Cradle to farm gate	A basket of products (Farm approach): 1 kg FPCM + the respective amounts of co-products live animals and crops	Detailed questionnaire to farmer Site-specific data (measurement of field emissions)	System expansion

MFS = Mixed farming system; SS = Specialised system; FPCM = kg fat and protein corrected milk

The selected FU is a basket of products including milk, live animals sold for meat and crops (farm level). Thus, a farm-level approach is applied which takes into account all products of the farm to allow a fairer comparison of the environmental impacts of mixed and specialised farming systems. The allocation method is based on a consequential approach (system expansion). As mentioned above, a consequential approach is more suitable for studying changes in production.

The primary data for the compilation of the LCI is collected using a detailed questionnaire for a real farm located in the Alexandria region of Romania. Background data on input manufacturing and emissions (mineral fertilisers, pesticides, cleaning products and medicines, electricity, etc.) are extracted from the ecoinvent database. Field emissions of CO_2, CH_4 and N_2O are estimated through site-specific measurements.

The Life Cycle Impact Assessment (LCIA) is carried out using the ReCiPe midpoint (H) method (Huijbregts et al., 2017) with openLCA v.1.11.0 software in order to quantify the environmental impacts and to identify the main hotspots. Midpoint indicators are recommended to represent impacts stemming from agricultural production because they are easily understandable for communicating results, and because a limited number of indicators can effectively summarise relevant information (Mouron et al., 2006; Tendall & Gaillard, 2015).

CONCLUSIONS

In this study, the most relevant applications of LCA to MFS have been analysed. Our review revealed the complexity of conducting LCA of these systems and comparing relative studies due to the multifunctionality, the generation of multiple co-products and interactions between different farm components, as well as the use of different methodologies and approaches.

LCA of MFS should be conducted by considering the different interactions and synergies in the MFS at the whole farm level rather than at the product level. Stakeholder participation and real data are needed to carry out evaluations at the regional level, and thus, propose optimization strategies for the design of public policies aiming at promoting these systems under good management to achieve greater sustainability.

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RESEARCH ON DIFFERENT TYPES OF PROTEIN IN COW'S AND SHEEP'S MILK ACCORDING TO DIFFERENT INFLUENCING FACTORS (SEASON, FEED RATION, BREED, PHYSIOLOGICAL CONDITION)

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Abstract

This paper focuses on the dynamic of milk protein in cows and sheep and explores the factors that influence milk protein content. Milk protein plays a crucial role in determining the quantity and quality of milk products, and therefore, various studies have been carried out to investigate the factors that influence milk protein content. To achieve this, we plan to monitor several farms raising different breeds of cows and sheep over a period of time to determine the changes of protein content. The breeds of cows that will be monitored include Holstein, Brown, and Romanian Spotted breeds. While the breeds of sheep that will be monitored have not yet been determined. Previous research has shown that optimizing the nutrition and management of cows and sheep can increase milk protein content. However, further research is necessary to better understand the complex dynamic of milk protein in both species and to develop best practices for improving milk production and quality.

Key words: *cow, milk production, protein, Romania, sheep.*

INTRODUCTION

Milk is commonly defined as a whitish liquid produced by the mammary glands of female mammals after parturition. Physiologically, it is a complex biological fluid that contains a precise balance of essential nutrients such protein, fat, lactose and minerals. The main function of milk is to provide a source of nutrition to support the growth and development of new-borns in mammals (Martin et al., 1999). Milk has taken on a sacred meaning in some ancient cultures, such as Egypt, Iran and India, due to its close relationship with the cow. Before the scientific revolution and industrialization in the 19th century, the production of dairy products such as fermented milk, butter, and cheese already played an important role in human life (Konte, 1999). Nowadays, the dairy industry aims to meet the demands of consumers who are seeking innovative products with consistent quality. Therefore, it must harness the full potential of this seemingly simple but complex raw material. To succeed in the production and sale of dairy products derived from sheep and cows, it is crucial to have access to accurate information on the composition and physicochemical characteristics of the milk from these animals. This data is essential for the effective development of cattle and sheep supply chains and for the commercialization of their products. Sheep milk is used exclusively for cheese production, in order to ensure consistent quality and yield of cheese, particularly the levels of fat and protein, as these parameters have a significant impact on cheese yield (Pellegrini et al., 1997). Therefore, given the scarcity of available data on the variation of protein content in sheep's milk according to various factors, we conducted a bibliographic search to establish links between these variables and those of cow's milk. This research serves as only a preliminary introduction to our study, which will be conducted in a second phase.

MATERIALS AND METHODS

During the initial phase of preparing this review, the topic to be addressed was selected based on the work of several authors. After analysing the existing literature, it was decided that a review article was necessary to gather the scattered information in the current scientific literature on the variation of protein in cow and sheep milk according to several extrinsic and intrinsic factors. The following scientific databases were used to search for relevant articles using appropriate keywords: Web of Science, Science Direct, Scopus, and PubMed. Criteria were established for the inclusion of each publication in this review, prioritizing the most recent and relevant articles for the topic being addressed. The final version of this article includes references dating from 1987 to 2020, with particular attention given to publications from the last 15 years, which represent over 50% of the selected sources.

RESULTS AND DISCUSSIONS

1. Factors influencing milk production

As reported by several authors, the composition of milk varies according to various factors.

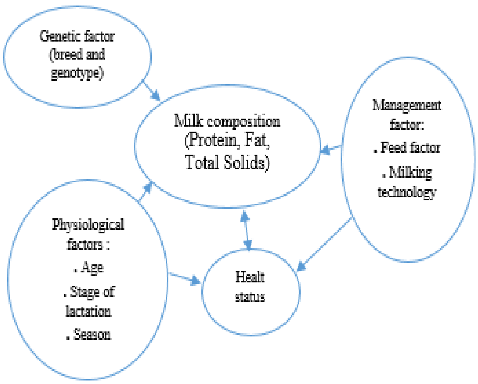

Figure 1. Factors that influence milk quality

Genetic factor

Several studies (Bencini & Pulina, 1997; Bencini, 2001) have established that there was a significant influence of breed on the chemical composition of sheep and cow milk. Considerable variations in milk production have also been observed between and within the same breed. According to Giambra (2011), selection can be used to improve milk production. In fact, selection for milk production has resulted in the creation of dairy breeds and sheep that produce more milk than meat or wool breeds.

For instance, the Awassi dairy breed is capable of producing up to 1000 liters of milk during a lactation period, while the Dorset Poll, a meat-producing breed, only 100 to 150 liters of milk per lactation. There is an inverse correlation between milk production and the concentration of fat and protein in milk. As animals produce more milk, the fat and protein content tends to decrease. This trend is observed not only between more or less productive dairy breeds, but also within a herd, among animals producing more milk, as well as in the same animal producing at different levels throughout its lactation (Casoli et al., 1989) This same author examined the milk composition of 12 breeds of sheep and reported a significant variation in the concentration of fat, ranging from 4.6% in Kurdish Iraqi ewes to 12.6% in Dorset ewe treated in America. The concentration of proteins was less variable, ranging from 4.8% to 7.2% in Armenian Corriedale sheep.

Nutritional factor

The quality and the quantity of consumed food, as well as the amount of ingested water, are key factors that have a significant impact on milk production. According to observations reported by (Saley & Steinmetz, 1994), healthy cattle and sheep with adequate nutrition produce more milk.

Additionally, higher quality nutrition can lead to slight increase in protein and casein content in milk.

Lactation stage and rank

According to research conducted by Casoli et al. (1989), the level of total solids, protein, lactose, and fat in milk tend to gradually decrease during the first few months and towards the end of the lactation period. Data gathered from literature suggest that the peak of milk production generally occurs the second or third lactation period.

Health status

The animal's health status, particularly in the case of udder infection, has a significant influence on milk composition. Most parasitic disorders such as trypanosomiasis, gastrointestinal parasitism, and external parasitism have an impact on milk production.

Milk composition

Sheep's milk differs from cow's milk in its higher content of total solids and essential nutrients. Although the lactose content of sheep's milk is comparable to that of cow's milk, the levels off fat and protein are significantly higher. Therefore, the proportion of lactose to total solids is lower in sheep's milk compared to cow's milk, at 22 to 27% versus 33 to 40%, respectively. The physical properties of sheep's milk, such as its density, viscosity, refractive index, titratable acidity, and freezing point, are higher than those of average cow's milk, as reported by Haenlein and Wendorff (2006). The lipids present in sheep and goat milk also exhibit superior physical characteristics compared to those of cow's milk, although the differences vary depending on the ratios, as emphasized by Anifantakis (1986) and Park (2006). However, some reports indicate that the quality of the different milks does not show significant differences.

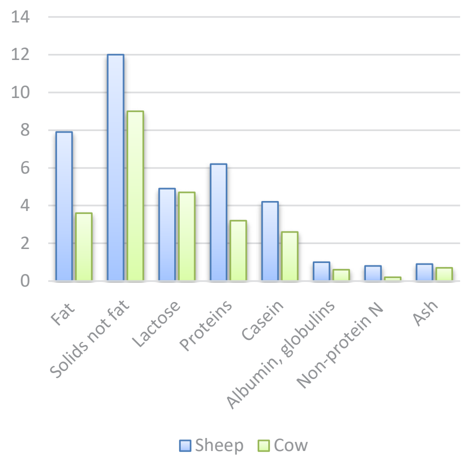

Figure 2. Average composition of basic nutrients in sheep and cow milk (%)

Lipids

Lipids are considered the most essential constituents of milk because they influence the economic nutritional, physical, and sensory properties of dairy products (Anifantakis, 1986; Park, 2006). The energy value and nutritional properties of milk are mainly attributed to the fat content, which also plays an important role in milk processing. The synthesis of milk fat occurs in the mammary epithelial cells, where lipids gradually accumulate and form inclusions that move to the upper part of the cell. The size of fat globules in milk varies from less 0.1 μm to around 18 μm, according to El-Zeini (2006). Mehaia (1995) reported a mean size of fat globules in decreasing order as follows: cow, sheep, and then goat. However, this observation is not entirely consistent with another research (Anifantakis, 1986). In sheep's milk, fat is mostly composed of triacylglycerols, representing about 98% (compared to 95% in cow's milk according to Jensen, 2002), which contain a large number of esterified fatty acids (Ramos & Juarez, 2011).

In addition to triacylglycerol's, sheep's milk contains other simple lipids such as diacylglycerols, monoacylglycerols, and cholesterol esters, as well as complex (Park, 2006; Haenlein & Wendorff, 2006).

The structure of triacylglycerols plays an important role in the rheological properties of milk fat and its behaviour during melting and crystallization.

Furthermore, the composition of triacylglycerols is interesting as it allows determining the origin of milk (Park et al., 2007). The chromatographic profile of sheep milk triacylglycerols present similarities with that observed in cow milk (Precht, 1992).

Moreover, sheep milk presents unique characteristics of fatty acid composition, with higher levels of linoleic acid, similar to those observed in goat milk, as well as a higher concentration of linolenic acid and polyunsaturated fatty acids overall.

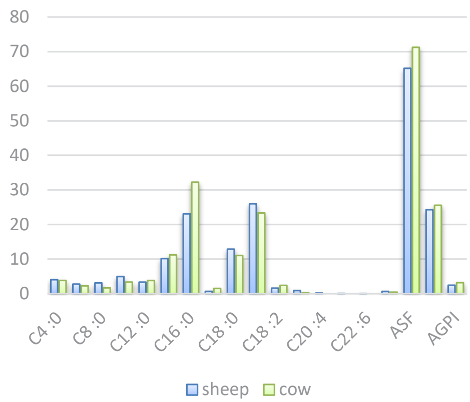

Figure 3. Fatty acids profile of cow and sheep milk (% total fatty acids)

Proteins

According to a study conducted by Park and colleagues in 2007, the most important proteins found in sheep's milk are similar to those found in cow's and goat's milk. Therefore, it is important to obtain simpler information about the composition for physiological and technological aspects. This same study shows that, in average, sheep's milk contains 5.8% protein, which is higher than that of goat's milk (4.6%) and cow's milk (3.3%). Nearly 95% of the total nitrogen in sheep's milk is protein-based, while the remaining 5% comes from other sources (Park et al., 2007). In contrast, goat's milk has a higher level of non-protein nitrogen and less casein than sheep's and cow's milk, which may result in lower cheese production resulting in a less structured texture (Guo, 2003). Sheep's milk, on the other hand, is particularly suitable for coagulation (Park et al., 2007). Several factors can influence protein content, such as lactation stage, season, age, and animal nutrition (Park et al., 2007). Furthermore, the specificity of small ruminants like sheep relies on the organization and mineralization of casein micelles, which are highly mineralized in sheep's milk (Pellegrini et al., 1994). There is also a small amount of a soluble protein with antibacterial properties called Lactoferrin. According to Kinsella and Whitehead (1989), there is a strong correlation between the major whey proteins, namely α-lactalbumin and β-lactoglobulin, and the nutritional value and functional properties of milk. Based on research conducted by Caboni and colleagues in 2019, sheep's milk is rich in lactoferrin and proteins with antibacterial and anti-inflammatory effects. This lactoferrin has also antioxidant, and anticancer effects.

According to research conducted by Zheng and colleagues in 2020, lactoferrin has the ability to attenuate oxidative stress in the hippocampus. Sheep's milk protein contains an important endogenous amino acid called proline, which plays a crucial role in the synthesis of arginine and polyamines, as well as in the activation of mTOR cellular signalling. This signalling initiates the protein synthesis process, including collagen. Proline and hydroxyproline are present in higher quantities in sheep's milk proteins.

According to some studies, the concentration of lactoferrin in cow's milk is inversely proportional to the somatic cell count (SCC) (Hagiwara et al., 2003; Rainard et al., 1982). Studies have also shown that lactoferrin concentration increases during natural or experimental mastitis (Chen et al., 2004; Rainard et al., 1982; Schmitz et al., 2004). In these cases, high levels of lactoferrin appeared to be due to increased synthesis by mammary epithelial cells and/or release by neutrophils present in inflamed tissues, suggesting that lactoferrin could be considered an acute phase protein in cow's milk.

Several factors influence lactoferrin and SCC concentrations. While SCC is regulated by cellular components transported by the bloodstream, lactoferrin is synthesized directly by the mammary gland (Molenaar et al., 1996). Increased somatic cells in milk are triggered by compounds from bacterial cells walls or bacterial metabolites, as well as endogenous components such as cytokines or complement components (Kehrli & Shuster, 1994). Increased cytokine levels during certain physiological processes could also influence lactoferrin synthesis (Baumrucker, 2005).

A study conducted on goats by Hiss et al. (2008) showed a correlation between high lactoferrin concentration and high SCC levels. These researchers suggested that increased lactoferrin levels could play a protective role for the mammary gland, as high somatic cell levels are often associated with the presence of subclinical infectious processes. Lehmann et al. (2013) found that when comparing IgG concentrations during lactation in two groups was not statistically significant, unlike lactoferrin. Thus, when the mammary gland is affected by processes that lead to an increase in SCC, IgG levels are not as strongly affected as lactoferrin levels. This observation could be explained in the mammary gland while IgG primarily comes from blood. Increases in IgG and SCC levels in milk have been widely documented in cases of natural and induced mastitis in cows (Caffin & Poutrel, 1988; Lehmann et al., 2013). It has been reported that levels of IgG, lactoferrin, and bovine serum albumin increase in response to mammary pathologies in cows (Levieux et al., 2002). However, in study of goats with subclinical mastitis, the SCC rate was high, but the IgG rate in milk was even lower than in healthy goats (Ferrer et al., 1997). Such contradictory differences could be related to many factors, such as species specificity, breed, source of infection, and intensity of mammary lesions, among others. Several studies, mainly conducted on cow's milk, have demonstrated a correlation between an increase in SCC and a decrease in milk production. High levels of somatic cell count negatively affect milk protein content, which can lead to a decrease in cheese quality (Barbano et al., 1991). The implication of SCC as an indicator of mastitis or hygienic quality may be different in sheep milk compared to other ruminants, given that in some countries such as France and the United States, values of up to 1 million cells/ ml can be considered normal (Raynal-Ljutovac et al., 2005), even in healthy sheep and goat milk, especially towards the end of lactation. Therefore, Riggio et al. (2010) reported that the SCC level may be high even when the sheep are not infected, suggesting that a healthy animal could be wrongly diagnosed as infected if the SCC level is exclusively taken into account.

a. Casein

Caseins are phosphoproteins that are synthesized in the mammary gland in response to lactogenic hormones and other stimuli.

They are secreted in the form of colloidal aggregates called micelle, which are responsible for most of the unique physical properties of milk (Melanie et al., 1999). Caseins are abundant in milk and play an important role in the dairy industry, making them of interest to biochemists who study them potentially as the most widely studied food proteins (Swaisgood, 2003). Casein represents the main class of proteins present in sheep milk, constituting between 76% and 83% of total proteins (Treacher & Caja, 2002). Among these proteins, the same types as in cow's milk are found, namely αs1-Cn, αs2- Cn, β- Cn, and κ-casein (Table 1).

Table 1. Comparaison of casein profiles for cow's and sheep's milk (%) (Balthazar et al., 2017)

Casein	αS1 casein	αS2 casein	β casein	κ casein
Cow	37	7	42	9
Sheep	6.7	22.8	61.6	8.9

Each of these fractions has a variable proportion in milk and exhibits polymorphism in most animal species (Vera et al., 2009).

The heterogeneity of caseins is determined by various factors, such as the presence of genetic variants, discrete levels of phosphorylation, variation in the extent of glycosylation of the κ-casein fraction, and the coexistence of protein with different chain lengths (Park et al., 2007).

Casein κ is one of the most extensively studied caseins, likely due to its crucial role in micelle stability and dairy processing. Unlike the others caseins (α and β), it contains carbohydrate residues within its structure (Fox & Mulvihill, 1992). In the presence of calcium phosphate, caseins form stable micelles in a colloidal phase that is balanced with the soluble phase of milk. This balance can be adjusted by altering factors such as temperature, pH, and the addition of salts. When lactic acid bacteria convert lactose to lactic acid, the pH of the milk decreases, leading to decalcification of the casein micelles. Alternatively, casein micelles can be destabilized using an enzyme, such as chymosine. Caseins and whey proteins are the main sources of protein in milk, rich in essential and non-essential amino acids, with a high biological value and good digestibility, absorption, and utilization.

b. Whey proteins

Whey proteins from sheep's milk represent between 17 and 22% of all the present proteins (Ramos and Juarez, 2011). The two main proteins found in whey are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), while immunoglobulins, serum albumin, and protease-peptones are present in lower concentrations. The latter are produced during the breakdown of β-casein by plasmin. The raw milk of various mammalian species constitutes an important source of protein for human nutrition. Table 2 indicates the total amino acid content (free plus protein-bound) per 100 g of milk from cows and sheep raised in Italy.

Although no qualitative differences were observed between the two types of raw milk, quantitative differences were observed. Glutamic acid and glutamine are the most abundant amino acids (approximately 21.6% and 19.6 %, respectively) in both types of milk. The amino acids leucine, lysine, and aspartic acid were also present in sufficient quantities (approximately 7 to 8%) in both types of milk, while proline was more abundant in raw sheep milk (9.3%) than in cow milk (8.9%). The other amino acids did not account for more than 5% of the total protein content, including tyrosine and serine. Furthermore, raw sheep milk had higher content of valine, threonine, and phenylalanine than raw cow milk. The amounts of methionine and cysteine did not exceed 4% of total protein for both types of milk, confirming a low content of sulphur-containing amino acids. In summary, the results indicate that both types of raw milk contain approximately 40% essential amino acids relative to total protein, thus confirming their protein quality. The results also suggest that the amino acid content varies depending on the mammalian species from which the milk is sourced.

Table 2. Amino acid composition of sheep and cow milk proteins, adapted from (Molik et al., 2012)

Amino acid	Cow	Sheep
Ile	4.01	4.22
Leu	8.81	7.91
Lys	7.79	7.47
Phé	4.67	4.76
Thr	4.87	5.06
Trp	-	-
Val	4.79	5.01
Ala	3.31	3.64
Arg	3.33	3.40
Cys	0.87	1.06
Gln	21.67	19.69
Gly	1.80	1.74
Pro	6.58	9.31
Ser	5.96	5.69
Tyr	5.00	5.04

Amino acids are the fundamental building blocks of all proteins, playing a crucial role in the structure and function of cells. However, amino acids also have other important biological functions. In particular, some amino acids are precursors to molecules such as antioxidants, which are essential for protecting cells against damage caused by free radicals. Glutathione, which is made up of the amino acids glutamic acid, cysteine, and glycine, is a naturally tripeptide that is found in most animals, plants, and microorganisms, and it plays various roles in cells, according to Meister and Tate 1976. These roles include its use as coenzyme, interaction with peroxides and free radicals, contribution to the formation of mercapturic acid, and its ability to protect membrane lipids and proteins. However, the presence of rapid turnover of the GSH tripeptide in some tissues suggests that is quickly broken down into its constituent amino acids and regenerated within the cell, as proposed by Meister in 1973. Researchers such as Meister in 1973, Griffith et al. (1979) have proposed that GSH also plays a role in amino acid transport, working in collaboration with the enzyme 7-glutamyl transpeptidase located on the outer surface of plasma membranes. 7-glutamyl transpeptidase is the only biological pathway currently known for breaking the γ-glutamyl bond of GSH. Additionally, it is possible to translocate glutathione out of cells (as reported by Bannai & Tsukeda in 1979, and Griffith et al. in 1979), and it can be utilized (metabolized) by tissues with high glutathione peroxidase activity, such as the kidney. The levels of glutathione were measured in the whole blood and plasma of lactating Holstein cows. Samples were taken from both the internal iliac artery and mammary vein to measure the arteriovenous differences across the vascular bed supplying the mammary gland. The results showed that plasma glutathione levels were very low and there was no significant arteriovenous difference in the levels of this tripeptide across the mammary gland. However, there was a significant disparity between the levels of glutathione in whole blood and plasma, with blood concentration being approximately 200 times high. Additionally, there was a significant arteriovenous difference in glutathione levels across the mammary gland. Studies conducted on living subjects have shown that insufficient levels of cysteine in plasma may limit milk protein synthesis. Using arteriovenous concentration differences and estimated blood flow, it has been calculated that the amount of glutathione absorbed by the mammary gland from whole blood is more than sufficient to explain the amount of cysteine secreted in milk. The mammary tissue of cows has a high activity of GTP cyclohydrolase, according to a study by Baumrucker and Pocius in 1978. This activity allows the tissue to use GSH to synthesis milk proteins. In vitro, studies have shown that the mammary gland has a low capacity for the absorption of cysteine and glutamate compared to other amino acids, according to research by Clark and al, 1978 and Derrig and al in 1974. Cysteine absorption from plasma is often in deficiency, with venous concentration higher than arterial concentration. The use of blood glutathione by the mammary gland as a source of amino acids could be the cause of these results.

Non-protein nitrogen

About 5% to 6.8% of the total nitrogen present in sheep's milk is considered non-protein nitrogen, consisting mainly of urea (45%), free amino acids (16%), creatine (2.4%), creatinine (1.7%), ammonia nitrogen (1%), uric acid (2.1%), and others compounds whose nature remains unknown (Park, 2006). Compared to cow's milk, sheep's milk contains higher amounts of urea and uric acid.

The remaining 20% of milk proteins include whey proteins such as β-lactoglobulin and α-lactalbumin as well as other proteins such as immunoglobulins, serum proteins, fat globule membrane proteins, transferrin, lactoferrin, β2-microglobulin, several enzymes, peptides, and proteolytic products. Protein levels and amino acid profiles vary considerably by species, as well as growth rate, and are influenced by genetic, physiological, nutritional, and environmental factors.

Water

Water is the predominant nutrient for all animals, and it is abundant in milk, representing approximately 88% of its composition. The amount of water in milk is regulated by the synthesis of lactose by secretory cells in the mammary gland.

Carbohydrates

Lactose is the main carbohydrate present in milk. It is formed when molecules of D-galactose combine in a β-galactoside 1,4 linkage. Its concentration in milk varies slightly, ranging from 4.5 to 5.2 g/100 g, and unlike fat content, its concentration cannot be easily modified by diet or by breed of animal. Lactic acid bacteria use lactose as a substrate during fermentation, which leads to the production of lactic acid, resulting in the characteristic texture and flavour of fermented products such as yogurts and cheeses.

Lactose plays a key role in the production of fermented milk as the amount of lactic acid produced by lactic acid bacteria depends on several factors, including bacterial strain, operating parameters, and the available amount of bacterial lactose. Additionally, the buffering capacity of milk also plays an important role in this process, as described by Fillion in 2006.

CONCLUSIONS

In conclusion, milk production for human consumption is subject to strict standards to ensure the health and safety of consumers. The quality and quantity of milk production can be affected by various factors, including race, species, health, nutrition, and age of the animal. Cow milk remains the most commonly produced and processed milk worldwide, but the milk from other mammals such as goats, sheep, and buffalo are of significant economic importance in certain regions, particularly those in the Mediterranean basin. Overall, continued research is necessary to understand the complex factors that affect milk production and quality in different species and to develop best practices for ensuring the availability of high-quality milk for human consumption. The knowledge of variability of sheep casein is still incomplete, which has been confirmed by the discovery of several new variants of casein during screening of milk samples from different breeds by isoelectric focussing (IEF). Therefore, this technique could be widely exploited for typing lactating ewes based on milk protein polymorphisms in a first step, followed by molecular methods. This allows for more complete picture of milk protein genes in sheep and consideration of milk protein variants/haplotypes in scientific breeding programs aimed at preserving biodiversity and/or improving dairy breeds for specific milk protein production.

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THE EFFECT OF THE SEASON ON THE PHYSICAL-CHEMICAL AND MICROBIOLOGICAL PARAMETERS OF MILK OBTAINED FROM BUFFALOES FROM THE FĂGĂRAŞ AREA

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Abstract

Currently, approximately 20,000 buffaloes are raised in Romania, of which 11% are found in the Făgăraș area. The present study was conducted to observe the effect of season on the components of raw milk obtained from buffaloes cows reared in this region. 320 milk samples were collected during the morning milking, during the grazing and stalling seasons, from 80 buffaloes cows in different stages of lactation. The fat, protein, lactose, non-fat dry matter (NFS), density and pH content were determined from the collected samples. Microbiological determinations mainly considered somatic cell count (NSC) and total germ count (NTG). The individual analysis of milk samples from buffaloes revealed significant differences in terms of the variation of these parameters, the researches carried out highlighted differences determined by the breeding system of the animals involved in the study, the feeding regime, as well as the reference season. Therefore, the results of the present research indicated that the season and stage of lactation influence the physicochemical and microbiological parameters of milk and could be minimized by better management practices.

Key words *: buffaloes, microbiological, milk, physico-chemical, season.*

INTRODUCTION

The Food and Agriculture Organization reports that worldwide buffalo milk in 2021 was 137 million tons, and represented 15% of total milk production obtained from domestic species (FAO, 2022). Interest and investment in obtaining buffalo milk in different countries has increased every year due to its unique taste and content in nutrients, being processed into many commercially important products such as butter, cream, assortments of hard and soft cheeses, yogurt and ice cream. The main physico-chemical characteristics of buffalo milk are defined by pH, density, fat content, protein, lactose and non-fat dry matter (NNF). Beside its physical and chemical properties, the microbiological quality is also quite important because in raw milk, microorganisms could multiply rapidly due to its rich nutrient content. High fat and calorie content, apart from dry matter, is considered the superior and distinctive property of buffalo milk. Changes in milk composition depend on many factors such as genetics, milking time, type of diet, age, udder hygiene and season. These factors greatly affect the quality and processability of milk into dairy products. Geographical area, climatic conditions and lactation period are known as seasonal changes and are listed as other factors affecting milk composition. The specialized literature is abundant regarding the composition of buffalo milk, but information about the influence of the animal breeding system, the feeding regime, as well as the reference season is limited. Currently, approximately 20,000 buffaloes are raised in Romania, of which 11% are found in the Făgăraș area. The total production of buffalo milk obtained in 2021 was 14,754 tons (INSSE, 2022). In this context, the aim of this study was to investigate the effect of the season on the physico-chemical and microbiological composition of milk obtained from buffaloes in the Făgăraș area , an important region with a dense population of buffaloes.

MATERIALS AND METHODS

The present study was carried out on 80 buffaloes in different stages of lactation, raised in 8 farms in the Făgăraș area, during 2022.

The buffaloes were kept on pasture between May and October, fed with discretionary green mass and concentrated fodder, in the amount of 2 kg/day/head, and between November and April in the shelter and fed with hay, in the amount of 8 kg/head/day, corn silage 20 kg/head/day and concentrated feed 2 kg/head/day. To ensure the necessary trace elements, the ration was supplemented with mineral salts in the form of lumps (briquettes for licking). The buffaloes were milked mechanically, with the help of the machine (installation) for mechanical milking at the drum. During the study, 4 milk samples were collected from each buffalo individually, during the morning milking, 2 samples during the grazing season (in May and August) and 2 samples during the stable season (November and December), in total 320 milk samples. Milk samples (250 ml/sample) were collected under aseptic conditions in sterile bottles, transferred to the laboratory and then analyzed. The analyzed parameters were represented by density, pH, fat, protein, lactose, ash, pH, nonfat dry matter content (SNF), somatic cell count (NCS) and total germ count (NTG).

Fat, protein, lactose, non-fat dry matter (NFS) and density were determined with an ultrasonic milk analyzer "EKOMILK-ULTRA". The pH values of the milk samples were determined by the portable pH-meter device "pH 3110".

Microbiological determinations mainly concerned: somatic cell count (NCS), total germ count (NTG). These parameters were determined using the following analysis methods: NCS through SR EN ISO 13366-2: 2007/AC/2011$_{(NR)}$; NTG through SR EN ISO 4833-1/2014$_{(AR)}$.

For the statistical evaluation of the milk parameters according to the season, the mean differences between the results of the collected samples were expressed according to the standard deviation (SD) and were interpreted using the ANOVA analysis of variance with the level of significance set at P≤0.05.

Figure 1. Mechanically milking buffalo in the household of the population

RESULTS AND DISCUSSIONS

The results of the physical analyzes performed on the milk samples taken during the grazing and stable period are presented in Table 1.

Table 1. Results of physical analyzes of buffalo milk (Mean with standard deviation X ± SD)

Season (n = 320)/Month of observation		Density (g/cm^3)	pH
Pasture (n = 160)	May - August (n = 160)	1.032 ± 0.01 ^{a}	6.74 ± 0.03 ^{a}
Stabilization (n = 160)	November - December (n = 160)	1,030 ± 0.01^b	6.83 ± 0.02^a

n = number of determinations
^{ab} the difference between mean values with different letters in the same column is statistically significant P ≤ 0.05.

The density of milk reflects its composition. The extraction of fat causes the density to increase and the addition of water to decrease it. It is a characteristic of the species and lies in the range of 1.026-1.034 g/cm^3. The density of buffalo milk normally varies between 1.027 - 1.033 g/cm^3. The current research produced results that support the conclusions of much of the previous work on buffalo milk, with milk density in the grazing season averaging 1.032 g/cm^3, and in the stalling season 1.030 g/cm^3, showing statistically significant difference (p≤0.05). The results of the present investigation are in agreement with the findings of some researchers who indicated that the season and stage of lactation influence the density of buffalo milk.

The density of buffalo milk collected from buffaloes in different stages of lactation from the Research and Development Station for Buffalo Breeding has statistical relevance between seasons ($p<0.05$), the lowest density value is recorded in the autumn season (1.027 g/cm^3), and the highest value is recorded in the summer of 1.033 g/cm^3 (Tătaru et al., 2019). Pece et al. (2009) stated that milk densities in the Romanian Buffalo breed were 1.034, 1.032, 1.032 and 1.033 g/cm^3, for the winter, spring, summer and autumn seasons. Romanian food regulations report that the density of raw buffalo milk must be equal to or greater than 1.028 g/cm^3.

Immediately after milking, the pH of the milk is neutral (due to the amphoteric characteristics of the proteins) or has a slightly acidic tinge, due to the preponderance of carboxylic groups or due to the presence of some acidic substances (citrates and carbonic acid). The metabolism of lactose causes the appearance of lactic acid and the decrease of pH. This acidification of milk can occur even within the mammary in the case of the development of some infections of the mammary gland determined by lactozofermentative bacteria (*Staphylococcus* sp., *Streptococcus* sp.), the milk immediately after milking has an alkaline tinge. Several studies have reported the pH of fresh buffalo milk, with large variations between individuals. In India, fresh milk obtained from Murrah buffaloes had pH values of 6.74 ± 0.08. In Italy a mean of 6.78 ± 0.03 was reported for fresh milk from Italian Mediterranean buffaloes, with marked seasonal fluctuations of 6.73 in August (summer) and 6.85 in December (winter) (Cockrill, 1974, Zava, 2011, Zava & Sanseniena, 2017, cited by Nikolau et al., 2022). In our study, the pH values varied between 6.74, during the grazing period and 6.83, during the stalling period, indicating that there is no infection in the udder and statistically, between the seasons, there were no significant differences in the values recorded ($p<0.05$). In a study carried out on the milk obtained on a farm with 190 Romanian buffalo, in different stages of lactation, between March 2020 and February 2021 (Nikolau et al., 2022), the highest pH value it was recorded in the spring season (6.64), and the lowest value (6.54) was recorded in the winter.

Table 2. Results of chemical analyzes of buffalo milk (Mean with standard deviation X ± SD)

Season (n = 320)/ Month of observation		Fat (%)	Protein (%)	Lactose (%)	SNF (%)
Pasture (n = 160)	May - August (n = 160)	7.14 ± 0.78^b	4.62 ± 0.22^a	4.54 ± 0.23^a	9.37 ± 0.35^a
Stabilization (n = 160)	November-December (n = 160)	7.53 ± 0.53^a	4.29 ± 0.27^b	4.26 ± 0.17^b	8.98 ± 0.27^b

(%) = (g/100 ml) ; n = number of determinations;
[ab]the difference between mean values with different letters in the same column is statistically significant $P \leq 0.05$.

One of the most important components of buffalo milk is fat. Fat content not only directly affects nutritional value and economy of milk, but also has an effect regarding the organoleptic properties. The fat content of raw milk is so important that milk processors tend to price it based on fat content. Also, the quality of dairy products such as cheese, cream and butter largely depend on the amount and quality of milk fat. The amount of fat in the milk, during the grazing period, recorded an average of 7.14% and during the stable period it was 7.53% statistically significantly higher than during the grazing period ($p \leq 0.05$). This increase in fat content during the stall period can be explained by the change in feed, the late period of lactation and the decrease in the amount of milk milked. The determined values are lower than the findings of Pece et al. in 2009, who stated that the fat in buffalo milk was 8%, 7.5%, 7.4%, and 7.8%, respectively, for winter, spring, summer, and autumn seasons. In another study carried out on buffaloes of the Romanian Buffalo breed (Nikolau et al., 2022), maintained under similar conditions as in the present study, the average fat measurements for all buffalo milk samples analyzed was 8.821 g/100 ml and ranged from 7.7 g/100 ml to 9.63 g/100 ml, with significant differences between the months of the year. The effect of season on milk fat percentage, which varied between 6.69-12% with a peak in the winter months, was found in a study of 120 samples collected from buffaloes in all four seasons (September 2009 - August 2010), from a farm located in Northern Transylvania (Mihaiu et al., 2010).

Another component of buffalo milk is the protein, which is responsible for the yield of milk processing into cheeses, internationally the amount of protein received compared to that of fat. Protein content of milk in grazing season (4.62%) and stable season (4.29%) showed statistically significant difference ($p \leq 0.05$). In a similar study (Tătaru et al., 2019), buffalo milk protein was significantly higher during summer (4.36%) and significantly lower during spring (4.00%) compared to the of autumn and winter and no significance was found for protein during the winter and autumn season. Pece et al. (2009) determined the protein in buffalo milk was 8%, 7.5%, 7.4%, and 7.8%, for winter, spring, summer, and autumn seasons, who stated that fat from buffalo milk, with the following mean values of 4.6%, 4.7%, 4.6%, and 4.6%, for the winter, spring, summer, and fall seasons. In our study, lactation stage was also a significant source of variation in protein percentage with a decreasing trend from early to late lactation (4.62-4.29%).

There is only one carbohydrate in milk, lactose, which influences its sensory properties, such as aroma and flavor, giving it a pleasant taste. In this study there was a significant difference ($p \leq 0.05$) in terms of lactose content between the milk obtained during the grazing period (4.54%) and that during the stall period (4.26%), this being being in balance with the protein. Other authors, in the qualitative evaluation of raw buffalo milk, (Tătaru et al., 2019), determined the highest values of lactose in spring (4.16%) and autumn (4.09%), and the lowest in summer (3.85%) and winter (3.94%). Pece et al. (2009), stated that the lactose content of the Romanian Buffalo breed milk was 4.8%, for all seasons. For lactose content, the global median was calculated to be 4.291 g/100 ml with values ranging from 4.00 g/ 100 ml to 4.62 g/100 ml. The average lactose in milk obtained from buffaloes in different stages of lactation, in the period March 2020 - February 2021, was calculated at 4.291 g/ 100 ml with values between 4.00 g/100 ml and 4.62 g/100 ml (Nikolau et al., 2022).

One of the important parameters when evaluating milk quality is the non-fat dry matter (NFS) content. The SNF in buffalo milk includes protein, lactose, vitamins, calcium and trace minerals. They contribute significantly to the nutritional value of milk. The results of the statistical analysis of the present study ($p \leq 0.05$) suggest that the milk obtained during the grazing period has a significantly higher SNF content (9.37%) than the milk obtained during the stalling season (8.98%). Other authors (Tătaru et al., 2019), showed that the solid substance without fat in buffalo milk, presents statistical significance ($p<0.05$) in winter compared to spring, autumn and summer, the lowest value of it is recorded in the winter season (8.86%), and the highest value is recorded in the summer (9.02%). The low content of SNF in milk, during the stalling period, is due to the lack of protein and nutrients in the diet, but also to heat stress. Standardizing the ratio of fat to non-fat solids in raw milk is essential for dairy production.

Table 3. Results of microbiological analyzes of buffalo milk (Mean with standard deviation X ± SD)

Season (n = 320) / Month of observation		NTG (cfu/ml)	NCS (no. cells/ml milk)
Pasture (n = 160)	May - August (n = 160)	140600 ± 68970[b]	82988 ± 3260[a]
Stabilization (n = 160)	November-December (n = 160)	164600 ± 170080[a]	84752 ± 4255[a]

n = number of determinations; ufc = Number of colony-forming units; [ab]the difference between mean values with different letters in the same column is statistically significant $P \leq 0.05$.

The presence of pathogens and microorganisms in raw milk and products made from inadequately heat-treated milk could pose a threat to public health. Table 3 shows the results of the microbiological examination of buffalo milk. We can thus describe that the microbiological analyzes show a significant increase ($p \leq 0.05$) of the total number of germs in the milk during the stall period ($164.85 \times 10^3 \pm 170.08 \times 10^3$ cfu/ml) compared to the grazing period where it was recorded an average of $140.60 \times 10^3 \pm 68.97 \times 10^3$ cfu/ml, the microbial load of raw milk being directly dependent on the hygienic conditions in the shelters during stalling. In other research conducted on buffalo milk, microbiological analyzes revealed an increase in total germ count (NTG) in winter (3.4×10^4 cfu/ml) compared to autumn, when the lowest NTG was recorded (1.1 x 10^4 cfu/ml), in 120 samples studied between August 2017 and May 2018 (Tătaru et al., 2019). Nicolau et al. (2022), in their study states that from the buffalo milk samples analyzed over the course of a year, NTG falls within European limits in only 4 months out of 12. These months are represented by March, where NTG has a value of 380 ± 131.5 x 1000 cfu/ml, in April with an NTG of 324 ± 67.9 x 1000 cfu/ml, in July with a value of 347.5 ± 217 x 1000 cfu/ml and in September with an NTG value of 419 ± 185 x 1000 cfu/ml.

Somatic cell count (SCC) is an internationally recognized parameter for evaluating milk quality and udder health. Determination of somatic cell count is an important parameter to be considered for quality control of raw buffalo milk and as a control parameter for udder health. In the case of somatic cells in buffalo milk, the result of the study (Table 3) has no significant difference between seasons (84752 ± 4.25 x 10^3 cells/ml in the stable period and 82988 ± 3.26 x 10^3 cells/ml in the grazing period).

In another similar study on 120 raw buffalo milk samples, the average somatic cell count was 1026.5 x 10^3, the lowest value was 9 x 10^3 cells/ml and the highest was 2044 x 10^3 cells/ml (Tătaru et al., 2019). Regarding the evolution of NCS during one year, the average value obtained was 304.85 x 1000 cells/ml, and it fluctuates, in September having the minimum value (170.5 ± 3.53 x 1000 cells/ml), following as in the other months of study to increase until reaching the maximum analyzed value, which is in February of 484 ± 104.65 x 1000 cells/ml (Nikolau et al., 2022).

CONCLUSIONS

The results of the present study indicate that the season and stage of lactation affect certain components of milk obtained from buffaloes in the Făgăraș area. The milk obtained during the grazing period presented a better quality both in terms of density (in the grazing season being on average 1.032 g/cm^3, and in the stable season of 1.030 g/cm^3), nutrients such as protein (4.62% in the grazing season and 4.29% in the stable period), lactose (4.54% in the grazing season and 4.26% in the stable period), SNF (9.37% in the grazing and 8.98% in the stable period), as well as the microbial load (140.60 x 10^3 cfu/ml, in the grazing period and 164.85 x 10^3 cfu/ml in the stable).

Seasonal variations result in a varied composition of buffalo milk, mainly due to the feeding of the animals. In the summer, the buffaloes feed on grass in the pasture, and in the winter they are fed with canned fodder. As mentioned above, feeding green fodder not only has a direct effect on the physico-chemical composition of milk, but can lead to a reduction in its microbial load.

The stage of lactation in buffaloes, being a physiological process, cannot be changed, instead good management practices such as adequate nutrition and maintenance during the stall period would improve the physico-chemical and microbiological properties of the milk obtained.

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EFFICIENCY OF GROWING OF CHICKEN BROILERS UNDER CONDITIONS OF COMPLIANCE WITH EU RULES OF WELFARE

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Abstract

The research focused on the effect of the application of the EU welfare rules on the technical-economic results achieved in a chicken broiler breeding farm. In this sense, three rearing halls identical in the usable area and technical equipment were studied, which were populated with day-old chicks Ross-308 following the densities imposed by the annual European funding program, as follows: batch Lm = 19 chickens/m^2 (mandatory minimum requirements); batch Lexp-1 = 17 chickens/m^2 (density reduced by 10% compared to the minimum requirements); batch Lexp-2 = 16 chickens/m^2 (density reduced by 15% compared to the minimum requirements). The level of production indicators was directly influenced by the density ensured, an aspect highlighted by the values calculated for the European Production Efficiency Factor and, respectively, for the European Broiler Index, which was much higher in the lot with only 16 chickens/m^2 (Lexp-2). The conclusion of the study was that the economic efficiency of chicken meat production farms affiliated with the annual European funding program strictly depends on the allocations received, as there are no price differences compared to farms that do not comply with welfare norms.

***Key words**: broiler hen, welfare, performance, profitability.*

INTRODUCTION

Estimates of global food consumption indicate increases of 50-60% by 2050 (Falcon et al., 2022) and especially for poultry meat, a food product appreciated for its moderate energy intake and high protein, vitamin, and mineral contents (Vukasovic, 2010). In the European Union, the demand for poultry meat has exceeded that of beef or mutton, even in countries where these meats are traditionally consumed (Devine, 2003; Marangoni et al., 2015); the exception is the USA, where although the consumption of poultry meat has increased, red meat still predominates, representing about 58% of the total meat consumption (Daniel et al., 2015).
In most countries, poultry meat is obtained within industrial production systems (Kryeziu et al., 2016; Usturoi et al., 2020), but with large differences in terms of the level of production achieved and especially economic efficiency, gaps printed by numerous factors with direct action they are indirect, starting from cultural/religious considerations (Devi et al., 2014) and ending with the economic situation of the respective country (Szollosi & Szucs, 2014; Tudorache et al., 2012). For example, the cost of production in EU countries is higher by approx. 45%/kg of chicken meat than that in Brazil, a phenomenon that can be compensated by diversification of production, orientation towards the products demanded by the current consumer market, and especially by qualitative differentiation (Magdelaine, 2003).
However, the industrial production of meat has also generated negative reactions from those interested in the welfare of birds, being accused of the lack of sustainability of the current production systems (Chodova et al., 2021; Poltowicz & Doktor, 2012), the too high density per surface unit and the deprivation of birds from access to the external environment (Curea et al., 2022; Eleroglu et al., 2015), the increasing incidence of specific diseases, but also the loss of some quality characteristics of the meat (Arrazola & Torrey, 2021; Custură et al., 2019; Wilhelmsson et al., 2019).

Against the background of these social problems, Directive 43/EC was adopted in 2007, which provides for the maximum stocking density for broiler chickens (Directive 2007/43/EC), which was also adopted by the legislation in our country; in essence, these legislative provisions limit the density of broilers to 33-42 kg/m^2, to ensure the welfare of the birds, but also to protect the environment by reducing the level of harmful gases produced by this category of farm animals (Applicant's Guide to Measure 14, 2021).

Starting from these considerations, our research focused on the degree of influence of the conditions imposed by the FEADR program measure 14, Subpackage 1b and 2b (reducing the density of birds by 10% and, respectively, by 15% compared to the density resulting from the application of the mandatory minimum requirements regarding the surface minimum allocated), on the technical-economic results achieved within a holding specialized in raising broiler chickens.

MATERIALS AND METHODS

To achieve the proposed goal, three batches of experience were created, differentiated by the number of chickens introduced per surface unit at the time of population, departing from the maximum norm of 42 kg/m^2 allowed in farms that access the annual European funding program, as follows: batch Lm = 19 chickens/m^2 (minimum welfare conditions); batch Lexp-1 = 17 chicks/m^2 (10% density reduction compared to the mandatory minimum requirements); batch Lexp-2 = 16 chickens/m^2 (15% reduction in density compared to the mandatory minimum requirements).

The biological material was represented by 61880 chickens belonging to the Ross-308 commercial hybrid (they came from the same hatching, from a hatchery located near the work unit), which were distributed in three rearing halls, according to the specified densities previously, respectively: lot Lm = 22610 heads; lot Lexp-1 = 20230 heads; lot Lexp-2 = 19040 heads.

The breeding of chicks was carried out following the principles of the intensive system (on permanent bedding), in halls identical in terms of useful surface (1198 m^2) and technical equipment, in which the same level of micro-climate factors was ensured (according to the provisions of the technological guide of the hybrid used); and the feeding of the chickens in the three batches was uniform, being given combined feeds with the following nutritional characteristics: the Starter recipe with 23.0% P.B. and 3000.5 kcal/kg E.M. (in the period 1-14 days), the recipe for Growth with 21.5% P.B. and 3100.7 kcal/kg E.M. (period 15-21 days) and the recipe for Finishing with 19.5% P.B. and 3200.5 kcal/kg E.M. (period 22-35 days).

The technical and economic analysis of the growth and utilization of broiler chickens in compliance with the EU welfare norms was made through the lens of specific indicators for this type of activity, calculated following the agreed methodology in poultry research:

- body weight = the chickens from the control pens were weighed individually, on the morning of the day they were delivered to the slaughterhouse (at the age of 35 days);
- increase in weight gain = the ratio between the weight difference of the chickens at the end of the period and that at the beginning of the period and the number of days of the period (g/head/day);
- herd losses = weekly mortalities were related to the initial herd of the week in question and accumulated over the entire growth period (%);
- feed conversion index = the ratio between the individual consumption of combined feed and the individual increase in weight gain (kg n.c./kg gain);
- European Efficiency Factor of Production (FEEP) = was calculated with the relationship:

$$\text{FEEP}= \frac{\text{Viability (\%) x live weight (kg)}}{\text{Age (days) x Feed conversion index (kg n.c./kg gain)}} \text{ x 100}$$

- European Broiler Index (IEB) = calculated according to the formula:

$$\text{IEB}= \frac{\text{Viability (\%) x Average daily gain (g/chicken/day)}}{\text{Feed conversion index (kg n.c./kg gain) x 10}}$$

- economic efficiency = represented the difference between total production expenses and total realized revenues. In the calculation of the revenues, the financial allocations granted per U.V.M. (large cattle unit) were taken into account, with a coefficient of 0.03 for birds and which are granted for reducing the density in the hall (by 10% and 15%, respectively, compared to a mandatory minimum), to reduce the level of noxes (by 30%) and for fuel excise. The main data obtained were statistically processed, calculating:the arithmetic mean ($\bar{x}$), the standard error of the mean (± sx) and the coefficient of variation (V%).

RESULTS AND DISCUSSIONS

Productive indicators. At the age of slaughter (day 35), body weight recorded average values of 1915.08 g in the case of chickens to which the highest population density was applied (group Lm), of 1940.66 g in chickens where the density was reduced by 10% (group Lexp-1) and 1994.42 g in chickens where the reduction in density was 15% (group Lexp-2). Percentage-wise, the weight differences between the batches were 1.32% between Lm vs. Lexp-1 and, respectively, 3.98% between the same control batch vs. Lexp-2; even between the two experimental groups there were differences in body weight, of 2.69% (Table 1).

Table 1. Productive indicators achieved by the chickens studied (n = 50)

Productive indicator	Statistics	Treatment		
		Lm	Lexp-1	Lexp-2
Body weight	Mean ± SEM (g)	1915.08 ± 52.22	1940.66 ± 39.17	1994.42 ± 27.90
	Variability (%)	19.28	14.27	9.89
Increased weight gain	Mean ± SEM (g/cap/zi)	53.57 ± 1.43	54.30 ± 1.08	55.83 ± 0.69
	Variability (%)	18.98	14.01	8.72
Livestock losses	Mean ± SEM (%)	1.85 ± 0,05	1.66 ± 0,03	1.45 ± 0,02
	Variability (%)	18.56	14.57	7.77
Feed conversion index	Mean ± SEM (kg n.c./kg spor)	2.129 ± 0.05	1.935 ± 0.04	1.779 ± 0.01
	Variability (%)	17.76	13.28	5.13

The increase in weight calculated for the entire studied period (1-35 days) was only 53.57 g/head/day in chickens from the control group (19 head/m^2), compared to 54.30 g/head/day in those from the Lexp-1 batch (17 heads/m^2) and of 55.83 g/head/day in the chickens from the Lexp-2 batch (16 heads/m^2), the difference between the Lm batch and the other two batches being 1.34% and respectively, of 4.05%; between the experimental groups there was a difference of 2.74%.

The lowest mortality rate, of only 1.45%, was in chickens with the lowest density per surface unit (group Lexp-2), followed by specimens from group Lexp-1 (-10% compared to the mandatory density) with 1.66% mortality and of chickens from the Lm group (minimum mandatory density) where the mortality was 1.85%. The differences between the batches were 0.19% (Lm vs. Lexp-1), 0.40% (Lm vs. Lexp-2) and 0.21% (Lexp-1 vs. Lexp-2), respectively.

The chickens from the Lexp-2 batch (16 heads/m^2) achieved the most favorable feed conversion index, of only 1,779 kg n.c./kg gain, followed by those from the Lexp-1 batch (17 heads/m^2) with 1,935 kg n.c./kg gain and of chickens from the Lm batch (19 head/m^2) with 2,129 kg n.c./kg gain.

The experimental factors also influenced the homogeneity of the studied characteristics, the coefficients of variation calculated for the Lm (V% = 17.76-19.28) and Lexp-1 (V% = 13.28-14.27) groups indicating a medium and even high variability, while in the Lexp-1 group production performances were much more uniform (V% = 5.13-9.89).

Production efficiency indicators. In the specialized literature it is stated that the production of meat obtained by broiler chickens is profitable only when the European Efficiency Factor of Production (FEEP) is at least 300 points.

The value of the European Production Efficiency Factor correlated with the level of productive parameters achieved by the chickens studied and which were influenced by the welfare conditions ensured, respectively by the density applied to the flocks (Table 2).

From this point of view, it turned out that the group of chickens where the mandatory minimum density of 19 heads/m^2 was applied (group Lm) achieved the worst performance during growth, hence the lowest EPEF, of only 252.24 points. In the next position were the chickens where the stocking was done with 17 head/m^2 (Lexp-1 group), with an EPEF of 281.77 points, while in the group with only 16 head/m^2 (Lexp-2) it was recorded the best level for EPEF, of 315.64 points.

Expressed as a percentage, the differences between the batches were 10.52% (Lm vs. Lexp-1), 20.12% (Lm vs. Lexp-2) and, respectively, 10.73% (Lexp-1 vs. Lexp-2); statistically, the differences between the groups were very significant, in each of the three comparisons performed ($p < 0.001$).

Table 2. European Production Efficiency Factor

Traits	Statistics	Treatment		
		Lm	Lexp-1	Lexp-2
Liveability (%)		98.15	98.34	98.55
Live weight (kg)		1.91508	1.94066	1.99442
Age at slaughter (days)		35	35	35
FCR (kg feed/kg gain)		2.129	1.935	1.779
EPEF (European Production Efficiency Factor)	Mean ± SEM	252.24 ± 5.81	281.77 ± 5.33	315.64 ± 4.15
	Variability (%)	16.28	13.38	9.29
	ANOVA p values	***Lm vs. Lexp-1: $p = 7.6 \times 10^{-14}$ ***Lm vs. Lexp-2: $p = 7.6 \times 10^{-14}$ ***Lexp-1 vs. Lexp-2: $p = 7.6 \times 10^{-14}$		

SEM - standard error of mean. ***highly significant differences between means for *p < 0,001*.

The European Broiler Index, although it is less used, this indicator allows the comparison of technical results from a poultry unit, but, like the EPEF, it does not highlight the economic aspect of meat production; so for example, if a very low stocking density is used, the gain in weight and implicitly the EBI, will register higher values, but the profit per unit of area will be correspondingly reduced, at the expense of the economic efficiency of the unit.

In the situation analyzed by us, the values resulting from the calculation for the EBI were at a level of only 246.96 points for the Lm lot (minimum welfare conditions), 275.96 for the Lexp-1 lot (density reduced by 10% compared to the minimum requirements mandatory) and of 309.28 points to the Lexp-2 batch (density reduced by 15% compared to the minimum mandatory requirements) (Table 3).

Table 3. European Broiler Index

Traits	Statistics	Treatment		
		Lm	Lexp-1	Lexp-2
Liveability (%)		98.15	98.34	98.55
Saily weight gain (g/day)		53.57	54.30	55.83
FCR (kg feed/kg gain)		2.129	1.935	1.779
EBI (European Broiler Index)	Mean ± SEM	246.96 ± 5.58	275.96 ± 5.08	309.28 ± 4.16
	Variability (%)	15.98	13.01	9.50
	ANOVA p values	***Lm vs. Lexp-1: $p = 7.5 \times 10^{-14}$ ***Lm vs. Lexp-2: $p = 7.5 \times 10^{-14}$ ***Lexp-1 vs. Lexp-2: $p = 7.5 \times 10^{-14}$		

SEM - standard error of mean. ***highly significant differences between means for *p < 0,001*.

From a statistical point of view, the differences between the groups were very significant ($p < 0.001$), highlighting also in this case the influence of the experimental factor tested by us (population density); expressed as a percentage, the differences between the batches were even greater than in the case of EPEF, the resulting levels being 11.74% (Lm vs. Lexp-1), 25.23% (Lm vs. Lexp-1) and 12.74% (Lexp-1 vs. Lexp-2).

The economic balance of meat production was calculated on the basis of the data obtained from the unit where our investigations took place and concerned the total production expenses and the income from the sale of chickens to a specialized slaughterhouse to which were

added the subsidies from affiliation of the farm to the annual European funding program.
In the batch where only the mandatory minimum density was ensured (Lm with 19 heads/m^2), the highest production expenses were recorded (51403.74 Euro/series), due to the higher number of chickens; the expenses from the mentioned lot were higher by 13.96% compared to those related to the lot where the density was 17 heads/m^2 (Lexp-1) and by 21.34% compared to the lot where the density was only 16 heads/m^2 (batch Lexp-2) (Table 4).
The income came from the sale of chickens at the slaughterhouse and the excise duty on diesel (both valid for all lots), from compliance with the noxes level (1.46 Euro/UVM for the Lexp-1 lot; 1.42 Euro/UVM for the Lexp-2 lot) and from compliance with the rules of density (3.19 Euro/UVM for the Lexp-1 lot; 4.79 Euro/UVM for the Lexp-2 lot). The revenues achieved on a growth series were 47648.82 Euro for the control batch, 46441.49 Euro for the Lexp-1 batch and 45161.95 Euro for the Lexp-2 batch.

Table 4. Economic balance of meat production

Specification		Treatment		
		Lm	Lexp-1	Lexp-2
Costs	Workforce	1502.87	1451.57	1425.93
	Sheet	737.38	737.38	737.38
	Hall preparation	82.06	82.06	82.06
	Hall heating	2618.38	2618.38	2618.38
	Electricity	923.26	923.26	923.26
	One day old chicks	8605.45	7844.62	7383.25
	Combined feed	36062.37	29745.79	26497.33
	Drugs	871.97	823.22	767.74
	Total	51403.74	44226.28	40435.33
Income	Chicks delivered live	39498.25	35882.17	34774.68
	Subpackage 2b (density) 3.19 Euro/UVM=10% discount 4.79 Euro/UVM=15% discount	-	1903.85	2696.38
	Subpackage 3b (noxe) 1.46 Euro/UVM=10% discount 1.42 euro/UVM=15% discount	-	871.35	799.34
	Diesel excise duty (Euro/litre/UVM)	8150.57	7784.12	6891.55
	Total	47648.82	46441.49	45161.95
Benefits		- 3754.94	+ 2215.21	+ 4726.62

Under these conditions, the highest net benefit (4726.62 Euro/house/series) was achieved by the chickens in which the stocking density was reduced by 15% compared to the mandatory minimum (batch Lexp-2), followed by the chickens where the density was 10% lower than the minimum (lot Lexp-1) with a benefit of 2215.21 Euro/hall/series; the batch of chickens where the minimum mandatory density was ensured for the chickens (batch Lm) ended the series with a negative balance, having losses of 3754.94 Euro/house

CONCLUSIONS

From the data regarding the production indicators of the production of Ross-308 chickens in compliance with the EU welfare standards, it turned out that the best results were in the batch where the stocking density was reduced by 15% compared to the mandatory minimum requirements (Lexp-2), and the weakest in the batch where the density was at the mandatory minimum level (Lm).
This state of affairs is also attested by the values obtained for the European Production Efficiency Factor (the score of the Lexp-2 batch was higher by 10.73-20.12% than the other variants tested) and especially by the values established for the European Broiler Index (the differences between the batch previously highlighted and the other lots were even higher, 12.74-25.23%).
From an economic point of view, the variant where the population density was reduced by 15% (lot Lexp-2) registered a double benefit compared to the solution with a 10% reduction in density (Lexp-1), while the lot with the

minimum mandatory density (Lm) posted losses on the growth streak.

In conclusion, it can be stated that the economic efficiency of chicken meat production in the case of farms affiliated with the annual European funding program depends on the subsidies granted, the size of which correlates with the self-imposed reductions for density and noxes. This situation can endanger the existence of poultry establishments since there are no incentive price differences between meat obtained under welfare conditions and where such rules are not respected.

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RESEARCH ON THE USE OF BIOFERTILIZERS IN MULBERRY CULTURE AND SILKWORM REARING

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Abstract

One of the objectives of the sericultural research is the reducing of research field was the reducing of chemical fertilizers quantity by applying some ecological agricultural practices, among which it can be mentioned, next to using extra-radicular fertilizing, also the biofertilizers of vesicular-arbuscular mycorrhize (VAM) type, in the form of commercial products obtained by biotechnologies of high biological performance. The biofertilization of VAM type aims to reduce or eliminate the chemical fertilization, the mulberry being a plant with a high consumption of mineral elements. Also, this type of mycorrhize stimulates the plants growing and development, having a role in soil remediation and nutrition improvement from soil-plant system.

***Key words**: mycorrhize, mulberry, silkworm.*

INTRODUCTION

The sustainable agriculture implies the using of agricultural practices which determine the increasing of useful productive capacity for all the agro-ecosystems by making capital out of the ecopedological and biological factors, in the conditions of maintaining the balance of respective agro-ecosystems. The sustainable agriculture is based on economic-ecological principles of avoidance of any form of environment pollution and of making profits (Bethlenfalvay & Schüepp, 1994; Bhale et al., 2018).

The sericiculture constitutes an agricultural activity which by its biotechnological characteristics can meet the requirements imposed by the practice of sustainable agriculture, the silkworms being extremely sensitive to a multitude of pollution factors.

The studies aimed the reducing of the quantities of chemical substances used in the technologies of mulberry exploitation, in particular for fertilization, by the approach of some alternative agricultural practices, among which lately it is mentioned the utilization of biofertilizers of vesicular-arbuscular mycorrhize (VAM) type, in the form of commercial products obtained by biotechnologies of high biological performance (Chikkaswamy, 2015).

As main economic effect, the emphasis of vesicular-arbuscular mycorrhize (VAM) presence in mulberry varieties created the premises of establishing the optimum doses of Endorize SOL and NPK chemical fertilizers, in accordance with the soil fertilization, reducing in this way the doses of chemical fetilizers recommended in the classic technologies of mulberry plantaions, and thus the technological costs (Babu et al., 2013; Baqual, 2013; Moorthi et al., 2016). Also, this type of mycorrhize stimulates the plants growing and development, having a role in soil bioremediation and nutrition improvement from soil-plant system (Pavankumar et al., 2020).

The mycorrhizal associations established those biofertilizers using, increase the absorption of mineral elements from soil and improve the poor absorption of some nutrients (Kumaresan et al., 2010).

The use of vesicular-arbuscular mycorrhize (VAM) determines the reducing of chemical fertilizers, in particular of those with phosphorus, stimulating in the same time the absorption of secondary mineral elements (Mg, Ca, S) and of microelements (B, Cu, Zn, Fe). The presence of vesicular-arbuscular mycorrhize is conditioned by the soil type, the host plant

species, the environment conditions and the agricultural practices used (Chakraborty et al., 2015; Greiss et al., 2003; Petkov et al., 2006).
The mycorrhize is a symbiotic association between the plants'roots and fungous, having a major role in many fundamental functions of plants, of which the most important being the nutrition with mineral elements from soil and the resistance to the environment conditions stress (Begum et al., 2019; Ghulam Hassan Dar & Pankaj Dunge, 2020). As the relationship between soil and plant is important for the agricultural production, thus the mycorrhize represents a soil-fungous-plant relationship of great interest for the development of new strategies in sustainable agriculture, the mycorrhize enabling the reducing of the chemical fertilizers quantity, also of pesticides and thus minimizing their negative impact on the environment (Abbasi et al., 2015).
The researches targeted the method of using Endorize SOL biofertilizer, the doses and its influence on technological parameters in mulberry plants (saplings and adult plants) utilized in silkworm feeding and on biotechnological parameters in silkworms.

MATERIALS AND METHODS

The method of vesicular-arbuscular mycorrhize using in mulberry cultivation proposed for homologated constituted the primary documentation for the development of testing methodology of biological fertilizers for mulberry cultivation. The product Endorize SOL, utilized as biofertilizer in the project, contains the beneficial flora of vesicular-arbuscular mycorrhize from Glomus genus - *Glomus mosseae*, *Glomus fasciculatum*, *Glomus occulatum*, *Glomus heterogama*, *Glomus microcarpum* și *Sclerocystis* sp.
The experimental device in field was realized in an uniform plot, the vegetal biological material being represented by cuttings and mature mulberry plants fertilized with Endorize SOL product during the period of starting in vegetation (April) in order to establish the mycorrhizal associations in 40-50 days, corresponding to the phenophase of intensive growth of shoots, when the consumption of nutrients from soil is maximum.
The experimental variants were the following:
V_0 - control - mature mulberry plants - unfertilized;
V_1 - mature mulberry plants - NPK fertilized, dose 240: 120: 120 kg a.s./ha;
V_2 - mature mulberry plants - fertilized with Endorize SOL, dose 25 mg/plant.
The determination of the production of mulberry leaves/bush for each experimental variant was made by harvesting the whole mulberry leaves from 3 specimens/variant and the experssion of production by the quantity of leaves per bush. Another agro-productive parameter was the weight of 25 leaves with/without petiole, as average sample for each variant.
The silkworm rearings were made in two series, spring - summer, during June, 1st - July, 5th, and respectively summer - autumn, during August, 15th - September, 15th. For the first rearing series the biological material was represented by silkworms from the simple hybrid Select, and for the second series, silkworms from Băneasa 75 (B75) race and from simple hybrid Select, were used.
Each variant of rearing had 3 repetitions obtained by the distribution of larvae resulted from 1 g silkworm eggs. The feeding was made with mulberry leaves harvested according to the experimental variants and given *ad libitum* to silkworms until cocoons have been formed.
The methodology to determine the influence of Endorize SOL product on the rearing parameters consisted in the individual weighing of 25 raw silk cocoons for each variant of rearing and the average value experssion for raw silk cocoon weight and shell cocoon weight (raw silk cocoon weight after extracting the chrysalis).
For the IIIrd and Vth ages were collected samples for veterinary health testing of silkworms from the experimental variants fertilized with Endorize SOL product.

RESULTS AND DISCUSSIONS

Results on the use of vesicular-arbuscular mycorrhize in producing mulberry cuttings

The mulberry cuttings inoculated with vesicular-arbuscular mycorrhize constitute mulberry planting material which respects the technical characteristics of quality and certification of mulberry rooted cuttings concerning the somatic parameters - root, stem, crown - and the biological authenticity of 100%

of the initial material. The mulberry planting material represented by mulberry cuttings inoculated with vesicular-arbuscular mycorrhize allows the application of some doses of chemical fertilizers reduced at ½ of the recommended doses, decreasing the risks of soil and groundwater pollution. These inoculated cuttings are intended for establishment of mulberry plantations for sericultural exploitation, respectively silkworm rearings and for establishment of plantations of grafted branches, as source of initial biological material. After using Endorize SOL product for mulberry cuttings' inoculation, it was obtained an increase of rooting percentage in Ucraina 107, China 32 and Olteni varieties, in proportion of 64.5-76%, compared to the control of each variety, fertilized according to the traditional technology. Also stands out the improvement of cuttings' quality parameters regarding the number of issued roots, the length of issued roots, the length of cuttings stems and the thickness at bundle, compared to the control obtained in the conditions of current technology application. For the elaboration of the reference for the method of using Endorize Sol product, there were compared the parameters provided in the technical regulations regarding the mulberry planting material for cuttings and rooted marcots, with the results obtained experimentally (Table 1).

The ecopedological data regarding the influence of Endorize SOL product and the NPK chemical fertilization on the development of the radicular system of cuttings are presented in Table 2.

Table 1. Biotechnological data in mulberry cuttings with VAM type mycorrhize

Mulberry variety	Standard stem length (cm)	Standard roots no./ length (cm)	Experiment results dtem length (cm)	Experiment results roots no./ length (cm)
Kokuso 21 mycorrhize	50	5 /10	131.33	5.55/13.00
Kokuso 21 NPK fertilized	50	5 /10	172.00	5.00/16.67
Ucraina 107 mycorrhize	50	5 /10	175.33	6.67 /18.50
Ucraina 107 NPK fertilized	50	5 /10	160.00	2.33/13.50
Ichinose mycorrhize	50	5 /10	158.67	7.67/15.00
Ichinose NPK fertilized	50	5 /10	186.67	4.33 / 8.00
China 32 mycorrhize	50	5 /10	148.67	4.40/18.00
China 32 NPK fertilized	50	5/10	142.67	5.00/14.50
Olteni mycorrhize	50	5 /10	170.00	4.33/16.00
Olteni NPK fertilized	50	5 /10	171.67	5.67/20.00
Ken Mochi - Control	50	5 /10	160.33	3.00/21.33

Table 2. The influence of biofertilizer Endorize SOL and of chemical fertilization on the development of cuttings radicular system

Variety/cutting's number	Length of cutting (cm)	Length of main root (cm)	Number of secondary roots	Total number of active roots	Total weight of active roots (g)
Kokuso 21 variety with mycorrhize					
- cutting no. 1	116	11	6	36	0.34
- cutting no. 2	98	-	5	43	0.92
- cutting no. 3	180	15	5	56	0.97
Average	**131.33**	**13.00**	**5.55**	**45**	**0.74**
Kokuso 21 variety NPK fertilized					
- cutting no. 1	160	12	7	83	3.08
- cutting no. 2	156	30	2	48	3.35
- cutting no. 3	200	14	6	92	4.82
Average	**172**	**18.67**	**5**	**74.33**	**3.75**
Ucraina 107 with mycorrhize					
- cutting no. 1	220	20	8	88	1.270
- cutting no. 2	200	17	4	46	0.870
- cutting no. 3	106	13	8	86	0.840
Average	**175.33**	**18.50**	**6.67**	**73.33**	**0.890**

Ucraina 107 variety NPK fertilized					
- cutting no. 1	170	11	2	38	1.87
- cutting no. 2	150	-	2	29	0.83
- cutting no. 3	160	16	3	35	0.77
Average	**160**	**13.50**	**2.33**	**34**	**1.16**
Ichinose variety with mycorrhize					
- cutting no. 1	220	24	13	93	1.16
- cutting no. 2	140	6	3	28	0.16
- cutting no. 3	116	7	7	38	0.34
Average	**158.67**	**15**	**7.67**	**53**	**0.55**
Ichinose variety NPK fertilized					
- cutting no. 1	165	-	6	66	0.83
- cutting no. 2	185	10	3	38	0.61
- cutting no. 3	210	6	4	50	0.69
Average	**186.67**	**8**	**4.33**	**50**	**0.71**
China 32 variety with mycorrhize					
- cutting no. 1	232	20	7	60	1.26
- cutting no. 2	117	16	3	24	0.22
- cutting no. 3	98	-	2	12	0.16
Average	**148.33**	**18**	**4.4**	**32**	**0.88**
China 32 variety NPK fertilized					
- cutting no. 1	170	22	10	78	2.57
- cutting no. 2	160	7	2	38	1.15
- cutting no. 3	98	-	3	27	0.19
Average	**142.67**	**14.5**	**5**	**47.67**	**1.30**
Olteni variety with mycorrhize					
- cutting no. 1	190	9	9	102	2.02
- cutting no. 2	160	21	3	32	1.14
- cutting no. 3	160	18	1	25	0.66
Average	**170**	**16**	**4.33**	**53**	**1.27**
Olteni variety NPK fertilized					
- cutting no. 1	200	10	4	48	1.32
- cutting no. 2	160	30	7	80	1.52
- cutting no. 3	155	-	6	32	0.92
Average	**171.67**	**20**	**5.67**	**53.33**	**1.25**
Ken Mochi variety Control					
- cutting no. 1	200	21	1	65	1.45
- cutting no. 2	100	22	-	28	0.19
- cutting no. 3	190	21	5	120	2.18
Average	**163.33**	**21.33**	**3**	**71**	**1.27**

Table 3. The agroproductive parameters specific to mulberry plantations for silkworm rearing

Experimental variant	Leaves production (g/bush)	Difference to the control (g/bush)	Weight of 25 mulberry leaves without petiole (g)	Difference from the control (g)	The unitary production of mulberry leaves (kg/ha)	Difference from the control (kg/ha)
V_0 unfertilized control	1466	-	59.0	-	11728	-
V_1 chemical fertilization	1770	+304	60.0	+1.0	14160	+2432
V_2 Endorize SOL fertilization	1873	+407	60.0	+1.0	14984	+3256

Table 4. The influence of chemical fertilization of NPK type and of biofertilization with Endorize SOL product on the biotechnological parameters of silk cocoons *Bombyx mori* L. sp.

Experimental variant	Ist Series - Select hybrid				IInd Series - B75 race				IInd Series - Select hybrid			
	Raw cocoon weight (g)	Difference from the control (g)	Shell cocoon weight (g)	Difference from the control (g)	Raw cocoon weight (g)	Difference from the control (g)	Shell cocoon weight (g)	Difference from the control (g)	Raw cocoon weight (g)	Difference from the control (g)	Shell cocoon weight (g)	Difference from the control (g)
V_0 unfertilized control	1.89	-	0.379	-	0.960	-	0.229	-	1.031	-	0.156	-
V_1 chemical fertilization	2.01	+ 0.12	0.523	+0.144	1.184	+0.224	0.243	+0.016	1.3069	+0.2759	0.371	+0.115
V_2 Endorize SOL fertilization	1.98	+0.09	0.463	+0.084	1.148	+0.188	0.235	+0.006	1.099	+0.068	0.240	+0.084

It stands out the developing in vegetation of the cuttings, the values of inoculated cuttings length exceeding 2-3 times the standard length of cuttings. However, the varieties China 32 and Olteni presented values below the standard for the number of roots. The control used to compare varieties, Ken Mochi, does not meet the standard for the number of roots, such that the cuttings of this variety, along with the 2 others mentioned varieties, will be replanted in the replanted field, to develop at standard parameters.

Results on the use of vesicular-arbuscular mycorrhize mature mulberry plants for silkworm rearing

The results obtained in the production determinations are presented in Table 3.

The experimental data show the positive values of the agroproductive parameters specific to mulberry plantations exploited for silkworm rearing:

- the production of leaves per bush has the maximum value of 1873 g/bush in the variant fertilized with Endorize SOL product;
- the weight of 25 leaves without petiole had values between 59-60 g, specific to Ucraina 107 variety in all variants;
- the production of leaves per hectare registers values significantly positive higher than the control, with 3256 kg/ha in the variant fertilized with Endorize SOL and with 2432 kg/ha in the variant with chemical fertilizers.

Results on the influence of the biofertilizătion with Endorize SOL product on the biotechnological parameters of silk cocoons

The biometric determinations highlighted the following aspects (Table 4):

- normal values regarding the raw cocoon weight for the Ist series of rearing, higher than the IInd series, and among the fertilized variants were observed positive diferences compared to the control, the highest value registering in the chemical fertilized variant, of 2.01 g/raw cocoon, but close in value to the variant fertilized with Endorize SOL product, of 1.98 g/raw cocoon;
- the values regarding the shell cocoon weight and respectively the silk percentage indicate the same maximum values in the chemical fertilized variant, of 0.523 g/cocoon, close in value to the variant fertilized with Endorize SOL product, of 0.463 g/cocoon; the differences from the control in the two fertilized variants, of 0.144 g and respectively 0.084 g for the Select hybrid, are close to the values of the same hybrid in the IInd series, of 0.115 g and respectively 0.084 g;
- in the IInd series of rearing the maximum values are registred for the raw cocoon weight in Select hybrid, compared to B75 race, in both fertilized variants;
- in the IInd series the shell cocoon weight presents higher values in the fertilized variants in Select hybrid, compared to B75 race, and the control presents higher values in B75 race compared to Select hybrid, which indicate a better use of the mulberry leaves from the fertilized variants by the silkworms of Select hybrid;
- comparing the data in both aspects, the cocon weight and the shell cocoon weight, in both rearing series the variant fertilized with Endorize SOL product presents positive values compared to the control, lower than the NPK fertilized variant, but the differences are not significant.

According to the results of veterinary health testing of silkworms in the rearing variant with leaves harvested from the varieties fertilized with Endorize SOL product, the following characteristics of health surveillance became evident:

- uniformly developed larvae, without anatomical-pathological changes;
- negative virological examination for polyhedral crystals in silkworms from B75 race and Select hybrid;
- mycological examination – sterile cultures in both samples;
- negative parasitological examination for *Nosema bombycis* in silkworms from B75 race and Select hybrid.

CONCLUSIONS

The fertilization with Endorize SOL bioproduct does not change the agro-productive parameters in mature mulberry plants and silkworms *Bombyx mori* sp., compared to the NPK chemical fertilization, what influences positively the sericultural economic results by reducing the production costs related to fertilization, having beneficial effects of ecological protection on soil.

The technological parameters of the silk cocoons obtained in the variant fertilized with Endorize SOL product presents positive values compared to the unfertilized control.

For the variant of fertilization with Endorize SOL product, the analysis bulletin concludes negative bacteriological, mycological and parasitological examination for the pathogens specific to silkworms.

The mycorrhize technology has a great potential of application in order to improve the productivity and to reduce the environmental problems associated with the excessive use of pesticides.

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STUDY ON THE WELFARE OF DAIRY COWS ON FARMS IN SOUTHERN ROMANIA

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Abstract

The study aimed to present the importance of welfare regarding the animals, more specifically dairy cows. Five farms from the south of Romania where taken in the study. In order to examinate the level of the welfare for these farms was used the system ANI 35 (Animal Need Index), system that has 5 groups in its component. The analysis period was represented by the livestock year 2021-2022. The main results prove the fact that in Romania, in the south area, the cows from the farms studied benefit of optimal and good welfare condition. However, there are groups of characters that can be improved, in particular type and characteristics of the floor and outdoor areas. The paper highlighted the strengths but also the weaknesses regarding the welfare in the dairy farms. Based on the results obtained, correlated with the results from the rest of the country the national authorities can develop welfare legislation and the farmers can see where to action in order to ensure for animals better conditions.

Key words: *ANI 35, dairy cows, Romania, welfare.*

INTRODUCTION

Animal welfare has always been an object of interest in animal husbandry, but in the past it was not known under this name. Our ancestors also paid attention to the cows resting place, watering, feeding and last but not least their health. All this without knowing the term of "welfare". After the introduction of the term welfare, several definitions have been given to it over time. However, all definitions require the following: disease prevention and treatment, appropriate nutrition, shelter, management and humane handling (Fraser, 2008), in order to ensure the welfare of dairy cows.

If in the past the welfare was perceived only as the absence of pain, injury or illness and immediate treatment of animals, nowadays the perspective changed. These are no longer enough, the people are concerned about the modern farming techniques more precisely by the intensive system (Rushen et al., 2008). In the present, the welfare must include suitable space for each age group, appropriate space at the feeding front and the possibility of the animals to engage in social interactions and express their natural behaviour. That "new tradition" in animal welfare started in 1964 with the publications of Ruth Harrison in *Animal Factories* and continued in 1965 with the measurement adopted by UK Government in the Brambell committee. As an example of interest of behavioural restriction, in a Brambell report we find the following sentence written: we must draw the line at conditions which completely suppress all or nearly all the natural, instinctive urges and behaviour patterns characteristic of actions…as found in the ancestral wild species and which have been little, if at all, bred out in the process of domestication (Brambell, 1965).

Since the subject is of interest to many international governments legislative norms were adopted in order to to establish welfare norms and at the same time prohibit certain practices. In 1978, The Swiss Animal Protection Ordinance states that cows raised in loose - housing systems should not exceed the lying stalls available and must be kept in a manner that will not interfere with their behaviour. Also, in the same period other European countries adopted similar animal welfare legislation that formed the base of European Union legislation.

In the same time, others are of the opinion that this information are secondary comparing with food safety, taste and nutrition (Weatherell et. al. 2003; Grunert et al., 2004). The UE Commission

conclude that must be understood at the level of the caretaker, in the moment when the farmer will understand that the productive level is closely related to welfare the desire to implement all measures will come naturally.
The main purpose of this paper is to observe how the welfare is perceived in Romania dairy farms, what the legislation provides and what are the benefits produced by this both for cows and for people.

MATERIALS AND METHODS

The present research is based on amount of data, obtained after observing five farms in southern Romania. In these farms there are cows belonging to the Holstein and Montbeliarde breeds.
Two systems are usually used to evaluate the welfare conditions: ANI 35 and ANI 200 (ANI - Animal Need Index). The difference lies from the fact that ANI 35 system evaluate more the environmental and microclimate conditions while the ANI 200 system focuses on health and freedom of movement.
In our paper, in order to evaluate the welfare for the farms from southern Romania was used the system ANI 35. In system ANI 35, the animals welfare is studied according to five groups factors:

- freedom of movement;
- social interactions;
- characteristics and type of the floor, outdoor surface type;
- lighting, air quality and noise;
- tending and maintenances conditions.

For each of the 5 groups of factors, a sheet is drawn up. ANI is calculated by summing the points awarded for each of the 5 groups of factors separately.
The data obtain were processed and interpreted in order to present as correctly as possible the situation regarding the welfare of the animals in the studied farms.

RESULTS AND DISCUSSIONS

In order to analyze and interpret the welfare level from all the five farms from southern of Romania we will present the data from each group.
Firstly, we will present the data regarding the freedom of movement of the cows.

Table 1. Freedom of movement evaluation

Freedom of movement					
Group of factors/No. of farm	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Minimum area available m^2/head	3	3	2.5	2	2.5
Rest area comfort	3	3	3	2	3
Comfort offered by the stand	0.5	0.5	1	0.5	0.5
The possible movements in the stand	1	1	1	0.5	1
Access to the paddock days/year	3	3	3	3	3
Access to pasture days/year	-	-	1.5	1.5	1.5
Total points	10.5	10.5	12	9.5	11.5

In Table 1, the highest score registered is at Farm 3, with a total of 12 points. At a very small difference is Farm 5, with only 0.5 points above, Farm 1 and Farm 2 have the same score, 10.5 (in these farms the cows do not have access to pasture) and the lowest score is registered at Farm 4, a score of 9.5 points. We should mention that all farms received 3 points for the access to the paddock, that means that the animals are having access minimum 270 days/year.
Also, only Farm 4 do not received max points for the comfort of the resting area, only 2 points, which means that the level of comfort is medium. Farm 1, 2, 3 and 5 were noted with In table 1, the highest score registered is at Farm 3, with a total of 12 points. At a very small difference is Farm 5, with only 0.5 points above, Farm 1 and Farm 2 have the same score, 10.5 (in these farms the cows do not have access to pasture) and the lowest score is registered at Farm 4, a score of 9.5 points. We should mention that all farms received 3 points for the access to the paddock, that means that the animals are having access minimum 270 days/year.
Also, only Farm 4 do not receive max points for the comfort of the resting area, only 2 points, which means that the level of comfort is medium. Farm 1, 2, 3 and 5 were noted with maximum points at this group of factors, 3 points, this means an increased level of comfort.

In the following we will present the results obtained after the second group of characters.

Table 2. Social interactions

Social interactions					
Group of factors/no. of farm	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Minimum area available m^2/head	3	3	2.5	0	3
Group structure	1.5	1.5	2	0	0.5
Manag. of young cattle	0.5	0.5	0.5	0.5	0.5
Access to the paddock days/year	2.5	2.5	2.5	2.5	2.5
Access to pasture days/year	-	-	1.5	1.5	1.5
Total points	7.5	7.5	9	4.5	8

According to the second table, Farm 3 registered again the highest score for the social interactions group. All farms were scored with 0.5 points for the management of calves and young cattle, that because are raising calves obtained only in their farms, but in separate boxes. Maximum points were obtained by all the farms for the access to the paddock, the cows having access more that 270 days/year.

The lowest score is counted at Farm 4 and the causes are next: 0 points for the area available per head (less than 6 m^2/head) and 0 points for group structure (the cows being maintained in a linked system).

Farms 1 and 2 are scoring again the same, like in the previous group, but in this case only 7.5 points. That situation is caused again by the fact that the cows from these two farms do not have the opportunity to pasture.

Further, in Table 3 will be presented the result after completing the forms for the next group.

Table 3. Type and characteristics of the floor and outdoor areas

Type and characteristics of the floor and outdoor areas					
Group of factors/no. of farm	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
The elasticity of the resting area	2.5	2.5	2.5	2.5	2.5
The degree of cleaning in the resting area	0.5	0.5	0.5	0.5	0.5
The risk of slipping in the resting area	1	0.5	0.5	0.5	0.5
The quality of the floor in the active area	1	0.5	0.5	0.5	0.5
The type and characteris tics of the paddock	1.5	1.5	1.5	0.5	1.5
The type of pastures	-	-	0.5	0.5	0.5
Total points	6.5	5.5	6	5	6

As we can see in Table 3, Farm 1 obtained the highest score at this group of welfare, 6.5 points, at that in the circumstation with no pasture. Farm 3 and 5 registered 6 points, Farm 2 - 5.5 points and Farm 4, the lowest score, only 5 points.

All the farms obtained 2.5 points for the elasticity of the resting area (that means a thickness greater than 60 mm of the straw layer). Also, same score for the degree of cleaning in the resting area, 0.5 points (in others words the resting places are clean).

For the floor quality only Farm 1 obtained 1 point, in this case, the floor is clean, with no risk and not generating foot diseases.

The rest of the farms obtained only 0.5 points, here we can find a floor with a medium potential risk and the cows can suffer foot diseases. Regarding the paddock, Farm 4 has one of medium quality and the others paddocks of good quality, paved.

Table 4. Lighting, air quality and noise

Lighting, air quality and noise					
Group of factors/ No. of farm	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Natural ilumina tion	1.5	1.5	1.5	1.5	1.5
Air quality	1.5	1	1	0.5	1
Air currents in the resting area	0.5	0.5	0.5	0.5	0.5
Noise level	0.5	0.5	1	1	1
Outside acces days/ year	2	1	0.5	0.5	0.5
Outside acces h/day	2	2	2	2	2
Total points	8	6.5	6.5	6	6.5

Regarding the quality of the air, level of noise and lighting the Farm 1 scored the highest, 8 points. Farm 2, 3 and 5 have equal score, 6.5 points, Farm 4 the lowest score, only 6 points. All the farms received 1.5 points for natural illumination, meaning the fact that the shelters are close but the animals are having a good natural illumination. Equal score for the outside access also, the cows having access more that 230 days/year. For the noise level, Farm 1 and 2 were scored with 0.5 points, because are equipped fans but noise produced by them is not very loud.

The last group is represented by the tending and maintenances conditions. Here, again Farm 1 received the highest score, 7 points. Compared with the others farms, Farm 1 scored 1 point for the hygiene for feeding, watering and accommodation, while the rest only 0.5 points. The difference is also from the health of the batches, Farm 1 received 1.5 points and the others only 1 point. (1.5 - a very good health condition, 1 - a good condition). At hoof health all farms received 1 point, meaning a good condition, with an incidence of injuries less than 5%.

Based on Figure 1 we can extract the information that all the farms recorded the highest score at group - freedom of movement. At the same time, the group with the lowest scores is represented by type and characteristics of the floor and outdoor areas, an important indicator that shows us that there is potential for improvements at this level.

Table 5. Tending and maintenances conditions

Group of factors/No. of farm	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Hygiene of accommoda tion, feeding and watering areas	1	0.5	0.5	0.5	0.5
Skin condition	1	1	1	1	1
Air currents in the resting area	1	1	1	1	1
Body hygiene of animals	0.5	0.5	0.5	0.5	0.5
Hoof health	1	1	1	1	1
Incidence of technopathies	1	1	1	1	1
The batchs health	1.5	1	1	1	1
Total points	7	6	6	6	6

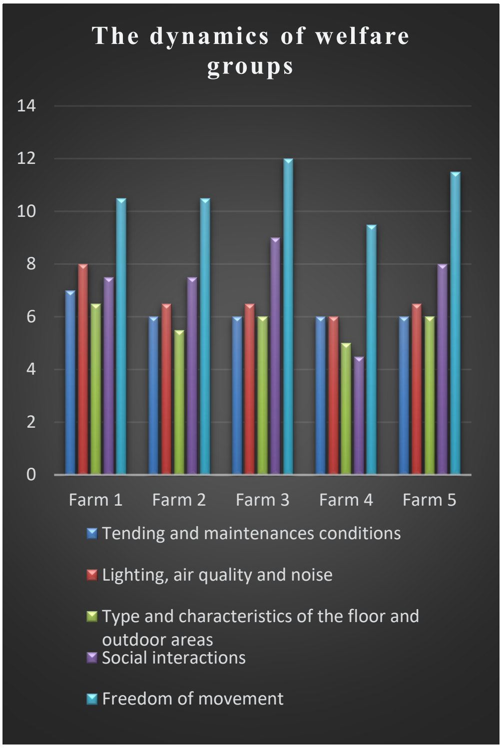

Figure 1. The dynamics of welfare groups

Figure 2. View from the paddock
where the animals have acces

After summarizing the groups scores, obtain the following results:

- Farm 1 - Total Score: 39.5 points;
- Farm 3 - Total Score: 39.5 points;
- Farm 5 - Total Score: 38 points;
- Farm 2 - Total Score: 36 points;
- Farm 4 - Total Score: 31 points.

According to these results we deduce the fact that the cows from Farms 1, 2, 3 and 4 have a optimal welfare and the cows from the Farm 4 have a complete welfare.

Figure 3. View from the rest area comfort

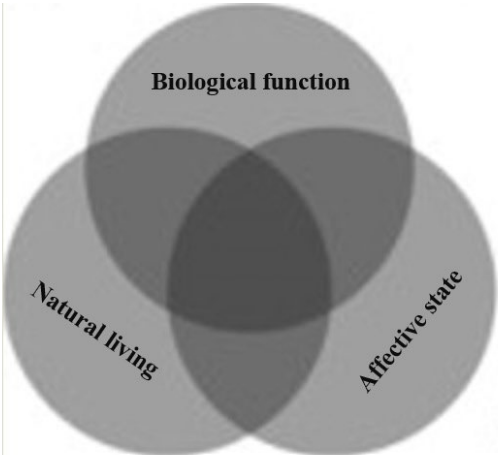

Figure 4. The welfare components

CONCLUSIONS

The concept of welfare arouses more and more interest in Romania, especially among farmers, but also among the national authorities. We note the existence of welfare norms and the constant interest in their improvement. According to the study we can find that dairy farms from the south of Romania ensure very good welfare conditions for the cows. At the same time, we cannot deny the fact that are possibilities to improve these conditions, particularly at the type and characteristics of the floor and outdoor areas and at tending and maintenances conditions.

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BODY CONFORMATION ANALYSIS THROUGH BIOMETRIC TRAITS OF AUBRAC CATTLE BREED

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Abstract

The purpose of this paper was to highlight the results of biometric measurements were performed on cattle of the Aubrac breed, exploited in Romania. Were measured a total number of 84 bovine, both adult females and bulls (12-18 months), in three farms in the region of Moldova. The highest recorded average for biometric parameters was: height at the withers (133.23 cm at females, 130.24 cm at bulls); rump height (140.78 cm at females and 130.46 cm at bulls); chest girth (202.15 cm at females and 204.22 cm at bulls); slantwise body length (159.64 cm at females, respectively 161.22 cm at bulls). Body conformation indices were calculated based on the obtained values. Cattle exploited within farm 2, show the most pronounced massiveness. Weight values between 553.21 and 603.13 were recorded in adult females and between 551.89 and 618.82 in bulls. As a general conclusion, we can state that the animals taken in the study, exploited in the three farms, present overall reports that denote a well-proportioned body development, within the specific morpho-productive type.

Key words: *beef cattle, biometric parameters, performances.*

INTRODUCTION

Aubrac cattle are a breed of cattle that originate from the Aubrac region of southern France. These cattle are known for their hardiness, adaptability to harsh conditions, and high-quality meat.

Physical Characteristics

Aubrac cattle are medium-sized with a weight of up to 1,000 kg for males and 650 kg for females. They have a light-colored coat, which can vary from yellow to dark red. They have a broad face, short but strong horns, and eyes that are usually surrounded by a black circle. Their body is muscular, with a well-rounded back and a deep chest (Mădescu et al., 2021).

Zootechnical Characteristics

The Aubrac breed is valued for its high-quality meat, which is tender and has a distinctive flavor. Additionally, these cattle are also used for milk production, as they have a high fat and protein content. They are also known for other characteristics, such as increased fertility, ease of calving, and adaptability to a variety of environmental conditions.

Aubrac cattle have a long lifespan, and they can graze on rough terrain that other breeds may find challenging. They are well adapted to living in harsh, mountainous regions and can thrive in forested areas. Due to their hardiness and adaptability, Aubrac cattle are often used in crossbreeding programs to improve the adaptability and hardiness of other breeds.

Popularity

Aubrac cattle have gained popularity in recent years outside of France, particularly in Europe and North America. The breed is known for its excellent meat quality and adaptability to various environments (Dransfield et al., 2003). Aubrac cattle can be found in several countries, including the United States, Canada, and the United Kingdom.

In recent years, the Aubrac breed has begun to gain popularity among Romanian farmers due to its special characteristics and superior meat quality. Several Romanian breeders started importing bulls and cows from France to improve their existing herds.

Body conformation is one of the main criteria for evaluating cattle from a zootechnical and economic point of view (Soulat et al., 2016). The term body conformation in cattle refers to the overall external appearance of the examined animal with reference to the development of each body region separately (Castilhos et al., 2018).

In cattle, body conformation differs depending on the direction of reproduction (meat, milk or mixed production). The analytical examination is based on the evaluation of each region of the body separately, in correlation with the development and functioning of the whole organism (Fonseca et al., 2017).
The synthetic examination is based on the evaluation of the animal as a whole, in correlation with its general development, harmony and proportionality of the whole organism (Stimbirys et al., 2016).
It is recommended that in each assessment of body conformation, the analytical examination should be completed with the synthetic examination, which consists in assessing the proportional development of all body regions, as well as how they merge with each other, depending on the morpho-productive type
The synthesis exam can be done by: free method; points method; body measurement method (biometric measurements)
By performing the synthesis test, it is possible to follow the way in which the animal develops in a certain time interval (for example from calving to maturity) and comparisons can be made between the characteristics of the breed.

MATERIALS AND METHODS

Body dimensions can be determined with various measuring instruments. Tools used: the zoometer for large body dimensions, the compass for small body dimensions, the metric tape for perimeters, the weighing scale for body mass (Mădescu et al., 2022).
The position of the animal during the measurements must be in a forced quadrupedal position, with the head and neck oriented in the normal position. Measurements may be made when the animal is outside or in the shelter.

Length measurements

Oblique trunk length - measured from the anterior part of the scapulohumeral joint (point of the back) to the posterior prominence of the ischial tuberosity (point of the buttocks), on which occasion it can provide information on body development and body shape;
Horizontal length of the torso - represents the distance, in the horizontal plane, between the tangent verticals at the point of the back and the point of the buttocks;
Chest length (depth) - represents the distance between the point of the back and the maximum convexity of the last rib, gives us indications on the development of the thorax and body capacity.
All these dimensions can be determined with the zoometer or compass (Ismail Awad et al., 2016).

Figure 1. Biometric measurements of adult female Aubrac cattle

Width measurements

Chest width - is the distance between the most prominent points of the ribs, measured immediately behind the shoulders. This measurement provides data on chest development and is determined by the zoometer.
Chest width - is the distance between the scapulo-humeral joints, taking as landmarks the lateral protrusions of the upper humeral extremities; gives indications on the development of the previous train.
The width of the croup - provides information on the development of this region, the degree of muscular dressing and is determined in three points:
- at the hips and represents the distance between the external angles of the iliac bones;
- in the coxo-femoral joints and represents the distance between the most prominent points of the coxo-femoral joints;
- in the ischium and represents the distance between the two sciatic protuberances.

The width of the head (forehead) is the distance between the external points of the orbits and gives us indications on the morpho-productive type. All these measurements are determined with the zoometer or compass.

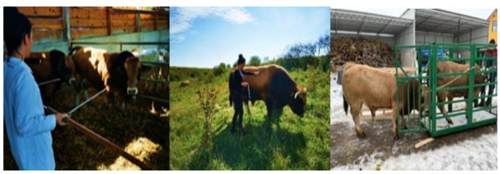

Figure 2. The measurement of the main body dimensions in the studied bovine populations

Perimeter measurements

The perimeter of the thorax is determined with the ribbon, immediately behind the shoulders and gives us indications on the development of the thorax, body capacity and the body as a whole (Paula et al., 2013).

The perimeter of the whistle is determined with the ribbon, on the left front limb, in the area where the whistle is thinner; it correlates with the degree of skeletal development.

Figure 3. Perimeter measurements

Interpretation and use of data - This is done by expressing the results of the measurements in absolute values, in relative values and in body indices.

RESULTS AND DISCUSSIONS

As part of the research, biometric measurements were performed on adult cattle, females and males aged between 12-18 months, from the Aubrac breed operated in 3 farms in Romania. The following biometric parameters were monitored: height at the withers, height at the rump, oblique length of the trunk, length of the rump, width of the forehead, width of the chest, width of the chest, depth of the chest, thoracic perimeter, perimeter of the whistle, width of the rump.

Based on the values obtained from the biometric measurements,the following body conformation indices were calculated: body shape index (I.f.c.%),chest depth index(I.a.t.%), massiveness index (I.m%), height difference index (I.d.i.%), skeleton index (I.o.%), head size index (I.m.c.%), cephalic index (I.c.%), thoracic index (I.t%), basin index thoracic (I.b.t.%), pelvis-pectoral index (I.b.p.%), robustness index (I.r.%), dactylo-thoracic index (I.d.t.%), whistle loading index (I.i.f.%).

Body indices represent the relative values, obtained by relating some dimensions to others, with which they are closely related anatomically and physiologically. These relationships of interdependence between the different body dimensions, serve to a greater extent to the overall appreciation, to the characterization of the type of co-formation and production of the animals.

The value of the same body index varies depending on the morpho-productive type, race, sex and age. In some cases, the value of some indices allows us to appreciate whether an animal has developed normally or not during the growth period.

Results regarding the average values of the main body dimensions, measured on the herds of adult Aubrac females, exploited in the studied farms.

The obtained results were expressed with the help of absolute values, which represent the real value of body dimensions and mass, expressed in physical units (cm or kg). These values give us indications on the general development of the animals and on the uniformity or variability of different characters within a population and on the assessment of the productive abilities of an animal.

Were measured 14 cattle from the Farm 1, 32 from the Farm 2 and 27 from the Farm 3.

In Table 1 are presented the average values on the farms, obtained after the statistical processing of the measurements performed.

Table 1. Average values of the main body dimensions measured on adult Aubrac females, raised on the studied farms

Monitored parameters (cm)	**Sample statistics**	**Farms**		
		Farm 1	**Farm 2**	**Farm 3**
		n = 14	**n = 32**	**n = 27**
Height at the withers	x	**129.10**	**133.23**	**135.46**
	± sx	0.81	0.97	0.91
Height at the rump	x	**137.28**	**139.14**	**140.78**
	± sx	0.53	0.84	0.52
Oblique length of the trunk	x	**149.45**	**159.64**	**157.91**
	± sx	1.12	1.28	1.01
Length of the rump	x	**43.23**	**42.41**	**45.72**
	± sx	0.42	0.48	0.47
Width of the forehead	x	**22.44**	**22.79**	**24.21**
	± sx	0.40	0.56	0.53

Monitored parameters (cm)	Sample statistics	Farms		
		Farm 1	Farm 2	Farm 3
		n = 14	n = 32	n = 27
Chest width	x	**45.36**	**48.34**	**47.11**
	± sx	0.82	0.87	0.52
Thorax witdh	x	**78.72**	**80.19**	**81.28**
	± sx	0.24	0.48	0.36
Depth of the chest	x	**95.22**	**96.63**	**98,42**
	± sx	0.32	0.59	0.33
Thoracic perimeter	x	**200.08**	**202.15**	**199.76**
	± sx	0.80	0.83	0.74
Perimeter of the whistle	x	**23.14**	**22.43**	**22.81**
	± sx	0.33	0.41	0.40
Width of the rump	x	**56.38**	**60.71**	**57.15**
	± sx	0.97	0.93	0.42
Weight (kg)	x	**552.21**	**602.13**	**581.60**
	± sx	8.01	12.82	9.45

The bovines exploited in Farms 2 and 3 have a more pronounced massiveness compared to those in Farm 1. Thus, the average body weights recorded for adult Aubrac cattle varied between 552.21 kg for Farm 1, 581.60 kg for Farm 3, and 602.13 kg for Farm 2.

The average values recorded within the three farms included in the study are represented for each biometric parameter analyzed. For example, the average values of withers height were obtained as 135.46 cm in Farm 3, 133.23 cm in Farm 2, and 129.1 cm in Farm 1.

Table 2. The average values of the main body indices, determined based on the biometric measurements carried out on adult female Aubrac cattle exploited in the studied farms

Monitored parameters	Sample statistics	Farms		
		Farm 1	Farm 2	Farm 3
		n = 14	n = 32	n = 27
I.f.c., %	x	**115.76**	**119.82**	**116.57**
	± sx	0.32	0.38	0.21
I.a.t., %	x	**73.78**	**72.52**	**72.66**
	± sx	0.22	0.48	0.30
I.m., %	x	**427.73**	**451.94**	**429.35**
	± sx	8.10	9.45	8.45
I.d.i., %	x	**106.33**	**104.44**	**116.57**
	± sx	0.08	0.55	0.19
I.o., %	x	**17.92**	**16.84**	**16.84**
	± sx	0.35	0.52	0.43
I.m.c., %	x	**33.49**	**31.83**	**33.75**
	± sx	0.78	0.76	0.59
I.c., %	x	**51.90**	**53.74**	**52.95**
	± sx	0.44	0.51	0.28
I.t., %	x	**82.67**	**82.99**	**90.90**
	± sx	0.76	0.89	0.78
I.b.t., %	x	**139.62**	**132.09**	**142.22**
	± sx	1.02	0.98	0.75
I.b.p., %	x	**84.98**	**79.62**	**82.43**
	± sx	0.25	0.56	0.32
I.r., %	x	**133.88**	**126.63**	**141.90**
	± sx	0.58	0.87	0.76
I.d.t., %	x	**11.57**	**11.10**	**11.42**
	± sx	0.49	0.53	0.23
I.i.f., %	x	**4.19**	**3.73**	**3.92**
	± sx	0.34	0.33	**0.78**

From the obtained values, it is observed that within Farm 2, higher average values were recorded for some indices such as the body format index (119.92%) and the massiveness index (451.94%). A higher value of the chest depth index is observed in the case of Farm 1 with a value of 73.78%, compared to Farm 3, where an average value of 72.66% was obtained. Within Farm 3, the highest average values were recorded for the height difference index (116.57%) and the robustness index (141.90%).

For example, it can be observed that the massiveness index recorded values ranging from 451.94% (Farm 2), 429.35% (Farm 3) and 427.73% (Farm 1). This is an index that illustrates the ratio between the animal's body weight and height, with higher values for meat breeds, and it also increases from birth to adulthood.

Additionally, within the study, biometric measurements were taken on adult males aged between 12-18 months from the Aubrac breed, which were raised on the same farms.

Table 3. The average values of the main body dimensions measured on the populations of Aubrac bulls raised on the studied farms

Monitored parameters (cm)	Sample statistics	Farms		
		Farm 1	Farm 2	Farm 3
		n = 2	n = 5	n = 4
Height at the withers	x	**125.08**	**130.24**	**129.52**
	± sx	1.63	1.05	1.24
Height at the rump	x	**127.31**	**133.62**	**130.46**
	± sx	1.52	1.35	1.43
Oblique length of the trunk	x	**148.96**	**161.22**	**155.62**
	± sx	1.84	1.62	1.53
Length of the rump	x	**36.74**	**38.73**	**37.99**
	± sx	1.58	1.12	1.43
Width of the forehead	x	**20.52**	**20.98**	**22.53**
	± sx	0.40	0.56	0.53
Chest width	x	**48.63**	**51.53**	**49.13**
	± sx	1.23	1.16	1.34
Thorax witdh	x	**80.72**	**82.99**	**83.85**
	± sx	1.72	1.96	1.76
Depth of the chest	x	**96.88**	**98.97**	**99.52**
	± sx	1.68	1.87	1.70
Thoracic perimeter	x	**201.92**	**204.22**	**200.74**
	± sx	1.24	1.53	1.39
Perimeter of the whistle	x	**24.78**	**23.63**	**23.26**
	± sx	0.89	1.01	0.98
Width of the rump	x	**57.01**	**59.98**	**58.14**
	± sx	1.49	1.58	1.46
Weight (kg)	x	**551.89**	**618.42**	**572.26**
	± sx	8.01	12.82	9.45

Additionally, the bulls raised on Farms 2 and 3 have a more pronounced massiveness compared to the individuals from farm 1 included in the study. Thus, the average body weights recorded varied between 551.89 kg (Farm 1), 618.42 kg (Farm 2), and 572.26 kg (Farm 3).

Regarding the oblique length of the torso, the bovine specimens from Farm 2 had the highest average of 161.22 cm, followed by the average of 155.62 cm from Farm 3, and the average oblique length of the torso recorded in the bulls from Farm 1 was 148.96 cm. Regarding the height at withers, the bulls raised on Farm 2 have the highest average height of 130.24 cm, followed by the bulls from Farm 3 with an average height at withers of 129.52 cm, and 125.08 cm for the males included in the study from Farm 1.

Regarding the thoracic depth, average values ranging from 83.85 cm for males from Farm 3 to 80.72 cm for males from Farm 1 were obtained.

Table 4. The average values of the main body indexes, determined based on the biometric measurements taken on Aubrac bulls raised on the studied farms

Monitored parameters	Sample statistics	Farms		
		Farm 1	Aubrac Butea	Aubrac Hălăucești
		n = 2	n = 5	n = 4
I.f.c., %	x	**119.09**	**123.78**	**120.151**
	± sx	1.12	0.89	0.85
I.a.t., %	x	**77.45**	**75.99**	**76.83**
	± sx	0.46	0.31	0.48
I.m., %	x	**440.51**	**474.83**	**441.83**
	± sx	10.55	8.48	9.15

Monitored parameters	Sample statistics	Farms		
		Farm 1	Aubrac Butea	Aubrac Hălăucești
		n = 2	n = 5	n = 4
I.d.i., %	x	**101.78**	**102.59**	**100.72**
	± sx	0.37	0.28	0.52
I.o., %	x	**19.81**	**20.44**	**17.95**
	± sx	0.42	0.23	0.37
I.m.c., %	x	**29.37**	**29.73**	**29.33**
	± sx	0.56	0.41	0.39
I.c., %	x	**55.85**	**54.16**	**59.30**
	± sx	0.36	0.43	0.41
I.t., %	x	**83.31**	**83.85**	**84.25**
	± sx	0.84	0.72	0.64
I.b.t., %	x	**141.58**	**138.36**	**144.22**
	± sx	1.24	1.03	1.06
I.b.p., %	x	**85.30**	**85.91**	**84.50**
	± sx	0.58	0.62	0.64
I.r., %	x	**135.55**	**126.67**	**128.99**
	± sx	0.94	0.93	0.84
I.d.t., %	x	**12.27**	**11.57**	**11.58**
	± sx	0.34	0.48	0.39
I.i.f., %	x	**4.49**	**3.82**	**4.06**
	± sx	0.29	0.21	**0.40**

Indices calculated based on the results obtained from biometric measurements can be particularly useful in assessing productive performance, especially in the case of cattle used for meat production. In the present study, it is observed that values of the bone index ranged from 20.44% in the case of bulls from farm number 2 to 17.95% in the case of males exploited within Farm 3. The bone index is an important indicator that can be a determining factor of carcass quality, influencing the meat-to-bone ratio.
Additionally, the average values recorded during the measurements carried out in the three farms included in the study can be observed. Regarding the robustness index, the bulls exploited in the first farm included in the study have the highest average of 135.55%, followed by the bulls exploited in Farm 3, where a robustness index average of 128.99% and 126.67% was recorded for males included in the study, respectively, the latter.
average being recorded in males taken from Farm 2. As a general conclusion, we can state that the animals included in the study, exploited in the three farms, present overall ratios that denote a well-proportioned body development, framed in the specific morpho-productive type.

CONCLUSIONS

As a general conclusion, we can say that the animals studied, exploited in the three farms, present overall ratios that denote a well-proportioned body development, framed in the specific morpho-productive type.
Biometric measurements are of particular importance in the study of beef cattle because through the exterior of the animals, we understand the totality of the external aspects of the animals' body that give us indications about their economic and zootechnical value. The scientific basis for assessing their economic value consists of the indissoluble link between function and form.
The body dimensions of Aubrac cattle can vary significantly between individuals, depending on the growth technology applied, the age of introducing young females to reproduction, the chemical composition of the administered diet, genetic factors, etc.

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THE IMPLEMENTATION OF MANURE DEGRADATED BY BLACK FLY LARVAE (*Hermetia illucens* L.*)* ON NATIVE LAYER PHASE CHIKENS EGG QUALITY

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Abstract

This study aims to determine the extent to which the utilization of manure flour resulting from the degradation of black fly (Hermetia illucens L.) larvae on egg weight, egg yolk weight and egg mass of native chickens the variables were egg weight, egg yolk and egg mass of layer phase native chickens. This study used a completely randomized design (CRD) consisting of 4 treatments and 5 replications in the form of degraded manure flour (MHD) of black fly (Hermetia illucens L.) larvae as follows: R0 = 0% MHD flour, R1 = 5% MHD flour, R2 = 10% MHD flour and R3 = 15% MHD flour. The results showed that the treatment had no significant effect (P>0.05) on the variable, so it could be concluded that the use of degraded manure flour (MHD) of black fly larvae (Hermetia illucens L.) with a level of up to 15% in egg-laying stage native chickens was added on feed formulations.

Key words*: eggs, larvae, manure.*

INTRODUCTION

Free-range chicken is a local chicken that is spread throughout the Indonesian archipelago which is often raised by breeders and rural communities as an effort to utilize their yards, fulfill nutrition and increase income. Poultry livestock, especially native chickens, is an alternative that is expected to provide animal protein for the community to fulfill the community's nutrition in the form of eggs. Egg quality is a general term to define external and internal quality. One of the factors that can affect the quality of native chicken eggs is the feed given to these livestock.

The productivity of laying hens is not only influenced by genetic factors but also by environmental factors (Dameanti et al., 2020) such as housing (Gustira et al., 2015). The nutrient content in the ration also greatly affects the quality of the eggs (Permana et al., 2020).

Manure is poultry faeces mixed with urine, and is the main food for a variety of insects in nature, including the larvae of *H. illucens* L. (black fly). The existence of insects in nature has indeed been created in such a way that they can play a role in the natural cycle of preparing nutrients from manure and of course they can produce a source of feed for livestock. Insects can biodegrade protein and other nutrient waste in manure into a protein-rich biomass that can be used as an alternative animal feed.

Larvae Degraded Manure (MHD) from (*Hermetia. illucens* L.) is an alternative feed ingredient as a protein source. MHD contains 51.15% protein (Manangkot et al., 2014). Furthermore, MHD is able to produce free-range chicken carcasses that are low in cholesterol (Rotinsulu et al., 2021). Manure Degradation Results (MHD) Black fly larvae (*Hermetia illucens* L.) are able to implement as a substitute for fish meal in rations of free-range laying hens on quality, cholesterol and triglyceride content in eggs and carcasses (Manangkot et al., 2014).

Starting from the above thoughts, a research has been carried out on "Implementation of Manure Results from the Degradation of Black Fly (*Hermetia illucens* L.) Larvae on the Quality of Layer Phase Village Chicken Eggs.

MATERIALS AND METHODS

This study used 60 female free-range chickens, Layer Phase, aged 6 months. The feed ingredients used in the study were yellow corn, concentrate and black fly larvae manure flour (MHD). The treatment uses a ration prepared based on the needs of free-range chickens. This study used 20 units of battery system cages with a size of 70 x 70 x 100 cm, equipped with places to eat and drink. Other equipment used is a scale, also equipped with an electric lighting device. The design used was Completely Randomized Design (CRD) with 4 treatments and 5 replications. (Steel and Torrie, 1994). as follows: R0 = 0% MHD flour, R1 = 5% MHD flour, R2 = 10% MHD flour, R3 = 15% MHD flour. Larvae Degraded Manure (MHD

Research procedure

The preparation was to provide MHD flour, which started with taking the manure, catching flies, until the stage where the flies were caged together with the manure for 8 days, then the manure was dried in the sun until it was dry, and ground into flour. Furthermore, the preparation of cages, places to eat and drink for free-range chickens is cleaned and sterilized with a disinfectant before use. The cages measure 50 x 50 x 75 cm each. Furthermore, before the chickens were put into the chicken coop, they were weighed to get the initial body weight. The chickens were randomized and put into the experimental unit.

Food and drinking water are given 2 times a day (morning and evening) *ad libitum*. Data on egg weight and yolk weight were recorded by weighing the eggs and yolks using a Petri dish as a container and an electric scale.

Research Rations

The composition of feed ingredients used to cover the nutritional needs of chickens used in preparing rations are presented in Table 1

Table 1. Composition of feed ingredients to make up the ration

Material Food	Proteins	Coarse fiber	Fat	Ca	P	EM
Corn[1]	8.60	2.00	3.90	0.02	0.30	3370
Concentrate[2]	30.00	7.00	2.00	12.0	1.50	970[3]
MHD Flour[4]	51.15	2.06	2.75	9.84	3.20	2940

[1]Composition Table Analysis Results (Scott et al, 1982)
[2]PT. Shinta Prima
[3]Results of Calculation of Metabolic Energy of Corn and MHD Flour
[4]Manangkot analysis results, 2014.

To be able to meet the needs of native chickens in accordance with the nutritional needs of layer phase native chickens, the composition of the research ration to be used, can be seen in Table 2 and the composition of food substances in the research rations in Table 3.

Table 2. Composition of Research Rations

Material Food	Amount			
	R0, %	R1, %	R2, %	R3, %
Corn	60	60	60	60
Concentrate	40	35	30	25
MHD Flour	0	5	10	15
Total	100	100		100

Table 3. Composition of ration food substances study

Food content	R0	R1	R2	R3
Proteins (%)	17.16	18.16	19.26	20.07
Coarse Fiber (%)	4.00	3.75	3.50	3.26
Fat (%)	3.14	3.18	3.22	3.25
Ca (Calcium) (%)	4.81	4.70	4.60	4.50
P (Phosphor) (%)	0.78	0.87	0.95	1.03
Metabolic energy (EM) Kkal/kg	2410	2509	2607	2705

Note: Calculation results of the composition of food substances.

Research Variables

1. Egg weight is a comparison between the total weight of the eggs produced (g) with the number of eggs produced (grains) or by the formula:

$$BT = \frac{\text{Total weight of eggs produced (g)}}{\text{Number of eggs produced (grain)}}$$

(North, 1984)

2. Egg Yolk Weight (g)
   The weight of the yolk is measured by weighing each yolk (North, 1984). The measurement is done by weighing the weight of the egg yolk (g) after being separated from the egg white.
3. Egg Mass
   Egg mass is the product of egg weight and HDP. The units are grams/head/day. Determination of egg mass Olgun et al. (2009)

As follows:

$$EM = \frac{\text{HDP X BT}}{\text{PP}}$$

EM : Egg Mass (g/ekor/hari)
HDP : Hen Day Production (%)
BT : Egg Weight (g/head/day)
RP : Research Period (day)

Data Analysis

Data were analyzed statistically using analysis of variance with Completely Randomized Design (CRD) (Steel and Torrie, 1994).

RESULTS AND DISCUSSIONS

Effect of Treatment on Egg Weight

Data from observations and calculations of the average egg weight of each treatment given during the study are listed in Table 4.

Table 4. Average Egg Weight (g/item)

Test	Treatment (g)			
	R0	R1	R2	R3
1	53.88	52.61	52.71	56.35
2	52.29	51.25	49.35	53.59
3	52.40	55.54	51.40	46.36
4	50.38	53.87	54.33	54.93
5	57.72	51.03	51.93	47.25
Total	266.67	264.30	259.72	258.48
Average	53.33	52.86	51.94	51.70

Based on analysis of variance on the use of manure flour resulting from the degradation of black fly larvae (*Hermetia illucens* L.) in free-range chicken rations, there was no significant effect ($P>0.05$) on egg weight. With the understanding that the use of manure flour resulting from the degradation of black fly larvae (*Hermetia illucens* L.) with levels of 5%, 10%, 15% in free-range chicken rations, does not cause a difference in egg weight.

The results showed that the average egg weight for each treatment ranged from 51.70 to 53.33 g. The results of this study are above the standards put forward by Sarwono (1995) that the weight of native chicken eggs ranges from 35.00-45.00 g. Egg weight is influenced by several factors, namely genetics, maturity stage, age, nutrients in the feed. Analysis of egg weight variance showed that treatment had no significant effect ($P>0.05$) on egg weight. This was due to the nutritional content in the research rations, especially protein and balanced energy, so that the effect of using manure flour resulting from the degradation of black fly larvae (*Hermetia illucens* L.) at a level of 5-15% did not have a significant effect on egg weight.

According to Latifah (2007) the size of the size of poultry eggs is strongly influenced by the content of protein and essential amino acids in the feed. The content of food substances in the rations of this study, including balanced protein and energy. That is, the content of nutrients has been fulfilled according to the needs of free-range chickens.

Effect of Treatment on Egg Yolk Weight

Observational data and calculation of the average egg yolk weight of each treatment given during the study are listed in the Table 5.

Table 5. Average Egg Yolk Weight (g/item)

Test	Treatment (g)			
	R0	R1	R2	R3
1	17.36	18.77	17.98	17.75
2	14.94	17.66	14.14	18.07
3	15.33	16.47	17.75	15.91
4	20.29	17.78	15.64	22.94
5	18.98	15.65	15.48	14.85
Total	86.90	86.33	80.99	89.52
Average	17.38	17.27	16.19	17.90

Based on the analysis of variance, it was shown that the use of manure flour resulting from the degradation of black fly larvae (*Hermetia illucens* L.) in free- range chicken rations showed no significant effect on free-range chicken egg yolk weight. The average egg yolk weight for each treatment ranged from 16.19-17.90 g. The results of this study are still above the standard put forward (Hartono et al., 2014), namely 12.83-16.00 g. Leeson & Summer (2001) stated that the weight of the yolk was affected by the quality of the feed. The results of this study, the weight of egg yolks is relatively the same. This means that the quality of the treatment ration, in this case protein and energy, is balanced, resulting in the same weight of egg yolks.

The effect of treatment was not significantly different on egg yolk weight. In this study, using a level of 5-15% manure flour resulting from the degradation of black fly larvae (*Hermetia illucens* L.) on egg yolk weight, the nutrient content in the rations was fulfilled.

Effect of Treatment on Egg Mass

Data from observations and calculations of the average egg mass of each treatment given during the study are listed in Table 6.

Table 6. Average Mass of Eggs (g/head/item)

Test	Treatment (g)			
	R0	R1	R2	R3
1	47.70	45.81	46.39	48.91
2	32.76	38.82	41.82	49.78
3	40.94	37.30	47.96	37.30
4	44.20	49.30	35.52	49.86
5	42.50	37.76	39.70	36.61
Total	208.10	208.99	211.38	222.45
Average	41.62	41.80	42.28	44.49

Based on the analysis of variance, it was shown that the administration of manure flour resulting from the degradation of black fly larvae (*Hermetia illucens* L.) at a level of 5-15% had no significant effect (P>0.05). This means that the use of 5-15% manure resulting from the degradation of black fly larvae in the ration is relatively the same. The average egg mass for each treatment ranged from 41.62-44.49 g/head/day. The results of this study are in the range stated by Leke et al. (2016) that the average egg mass is 40.10-44.94 g. So this study shows that R3 has the highest egg mass, namely 44.49 g. The average egg mass is related to egg production. High feed consumption will result in high egg production. The determining factors for egg mass are the number of eggs and the weight of the eggs. Egg weight is related to egg mass, where the pattern of increasing egg mass is in line with the growth pattern of mature follicles (yolk).

Egg mass is affected by feed consumption, because feed consumption is used for growth in order to reach an adult body while it is also used for egg production. Yolk weight of free-range chickens correlates positively with egg mass and egg size, the height and low mass of eggs depends on the feed (North & Bell, 1990). In the treatment ration, the higher the level of use of manure flour resulting from the degradation of black fly larvae (*Hermetia illucens* L.) is followed by an increase in protein and energy. That is, there is a balance between protein and energy in the research ration. So as to produce the same egg mass. If the egg mass increases, production also increases, conversely, egg mass decreases, egg production decreases (Nasikin et al., 2015). Egg mass is the correlation between egg weight and egg production.

CONCLUSIONS

Based on the results of data analysis and discussion that the use of manure flour resulting from the degradation of black fly larvae to a level of 15% produces the same egg weight, yolk weight and egg mass in native chickens. MHD can be used in free-range chicken rations as much as 15%.

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CHARACTERISTICS OF GROWTH AND WEAR OF HOOVES OF COWS RAISED ON PASTURE AND INDOOR CONDITIONS IN THE CENTRAL BALKAN MOUNTAINS

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Abstract

The paper deals with the study on individuals of two groups of cows, of red-white, wide-faced Montbeliarde and Simmental cattle breeds approved in Bulgaria according the qualities of their hoof horn and the impact of exogenous and endogenous factors on this process. Visual, metric and anatomically-topographical methods were used. Five measurements were made of the following indicators: length, width, total width, height and hoof angle of the thoracic and pelvic limbs of the studied animals during the indoor and pasture periods. Support point in cm² and the ratio of 1 kg live weight to unit support point in cm² were calculated. Pasture-raised cows, in both breeds, showed a higher coefficient (ratio) of a unit of support point compared to live weight, respectively a coefficient of 1,7576 for Montbeliarde breed or by 8.2% more and a coefficient of 1,3946 for Simmental breed or by 9.1% more. Mobility of cows affected critically to their ongoing health, productivity and longevity. Uniform growth of the hoof horn was determined by the equal distribution of cows' body weight over the distal limbs. Hoof growth was subject to seasonal fluctuations.

Key words: *breed, hoof horn, growth, pasture, length, width.*

INTRODUCTION

Biomechanical laws are constant for each species. In the biomechanics of cattle hooves, must be accepted the fact that the weight of the animals' body is distributed proportionally on all limbs (Hernandez-Mendo et al. 2007; Veremey et al., 2010; Homin et al., 2017; Hamzaev, 2019).
Cement floors, as a part of the mass technological process, lead to a higher growth intensity of hoof horn, with reduced hoof angle due to increased wear. Farmers must consider the conditions in the rearing room and the comfort requirements of the cows to prevent problems that are result of arising from hoofs. The time spent on hoof care combined with proper balanced feeding give results in the long-term period (Mochamadia & Khaglani, 2013; Marinov, 2016; Fedoseeva & Kolekniv, 2016; Longova et al., 2020).
Skin covering of cows' toes has changed into a kind of horned slipper in the process of phylogenetic development of cattle as species. Their hoof is a keratinized epithelium formed on the distal part of the limb. The derived layer of the epidermis, continuously produces a horn layer during the entire ontogenetic development of the individual (Muling & Creenongh, 2006; Kennedy et al., 2009; Rauibar et al., 2016).
Growth intensity of the hoof horn depends on various external and internal factors such as breed, sex, technology, sufficiency and dietetics of feeding, season of the year, physiological state, etc. (Cook et al., 2009; Kvochko et al., 2010; Shearer et al., 2013; Zemlyanukhina, 2016; Lomonov & Skorkina, 2020).
The objective of the present investigation was to study the growth and wear of hoof horn of cows of red-white, wide-faced Montbeliarde and Simmental cattle breeds during the pasture and indoor period in the region of Troyan in Central Balkan Mountains.

MATERIALS AND METHODS

The experiment was conducted from the end of May to the end of September in 2021, on the farm of Research Institute of Mountain Stockbreeding and Agriculture in Troyan, Bulgaria. By the method of analogues, two groups each of 10 cows were formed – 1st group of the Montbeliarde breed and 2nd group of Simmental breed. Cows were kept free-boxed in a barn during the winter, as each cow was provided with a bed covered with dry sand and straw. In the summer, the animals were raised on a pasture complex sown with traditional grass. During the experiment, the cows were fed in the same way, at a specific feeding rate. During the barn period, the animals were kept in a steel-concrete room with a cement floor.

Five measurements were made of length, width, total width, height and angle of the hooves of the thoracic and pelvic limbs of both groups of studied cows, at the beginning and end of the grazing period, and at the beginning and end of the barn period as well as using measuring instruments: retractable tape measure, measuring tape, plumb line and a protractor according to the methodology of Pesechkin (1976) for hooves characterization. Measurements were recorded in a protocol. Live weight of the studied animals was determined using an electronic scale with an accuracy of 0.01 kg. The support point in cm² and the ratio of 1 kg of live weight to a unit of support point in cm² were calculated. Visual, metric and anatomically-topographical methods were used. The data were processed by the methods of variation statistics using the program 'Statistica', version 10 and presented in Tables 1 and 2.

Table 1. Characteristics of the hoof horn, by breeds, cm

Size	Period			
	Pasture-raised		Barn-raised	
	At the beginning of the experiment	At the end of the experiment	At the beginning of the experiment	At the end of the experiment
Montbeliarde				
Front hooves of thoracic limbs				
Length	13.2±0.05	13.8±0.05	13.81±0.01	14.41±0.05
Width	6.2±0.05	6.7±0.05	7.4±0.05	7.6±0.05
Total length	13.2±0.05	13.5±.0.05	13.47±0.05	14.67±0.05
Height	6.2±0.05	6.4±0.06	6.3±0.05	6.5±0.05
Notched part angle	45±0.25	45±0.30	45±0.30	45±0.30
Back hooves of the pelvic limbs				
Length	13.22±0.04	13.56±0.07	13.72±0.23	14.52±0.03
Width	6.13±0.05	6.34±0.05	6.89±0.04	7.47±0.04
Total width	13.65±.0.04	13.88±0.05	13.72±0.05	13.97±0.02
Height	6.5±0.04	6.7±0.02	6.6±0.01	6.9±0.03
Notched part angle	54±0.30	54±0.30	55±0.05	55±0.04
Simmental				
Front hooves of thoracic limbs				
Length	15.02±0.05	15.64±0.04	14.31±0.04	15.78±0.03
Width	7.9±-0.04	8.11±0.04	8.5±0.05	8.84±0.05
Total width	14.87±0.04	14.53±0.02	15.1±0.05	15.32±0.05
Height	6.3±0.04	6.5±0.03	6.7±0.04	7±0.04
Notched part angle	44±0.01	44±0.02	44±0.04	44±0.05
Back hooves of the pelvic limbs				
Length	14.12±0.04	14.64±0.04	14.31±0.05	14.78±0.05
Width	6.94±0.04	7.11±0.05	7.35±0.05	7.94±0.05
Total width	13.53±0.08	13.89±0.06	14.24±0.05	14.83±-0.04
Height	6.5±0.03	6.8±0.04	6.6±0.02	6.9±0.05
Notched part angle	54±0.3	54±0.3	55±0.4	55±0.3

$P<0.05$*, $P<0.01$**, $P<0.001$***

Table 2. Live weight, support point and ratio between them

Period of raising	Number (n)	Live weight, g	Support point, cm²	Support point/live weight ratio, kg/cm²
Montbeliarde				
Pasture-raised	10	627.24±0.67	356.86	1.7576
Barn-raised	10	635.37±0.94	435.96	1.4574
Simmental				
Pasture-raised	10	644.12±1.33	461.86	1.3946
Barn-raised	10	658.46±1.95	513.69	1.2818

($P<0.05$*)

RESULTS AND DISCUSSIONS

In cattle, the anatomical-orthopedic features possess their own peculiarities. The hoof is modified skin of the limb forming a slipper around the third and fourth toe. It has a relatively thicker lateral wall compared to the medial one. In its notched part, the thickness of the wall is about 7 mm, and in the heel part, the same is about 5 mm (Marinov, 2016; Lomonov & Skorkina, 2020).

Growth and wear of the horn layer characterize the condition of the hoof horn in cows (Homin, 2017). Research has shown that the greatest rate of increase in hoof horn growth is observed during the grazing period in the order of 0.6 cm. According to experimental groups, hoof horn growth varies from 0.2 to 0.6 cm.

The studied cows from both groups demonstrated at the end of the pasture and barn period values of increase in hoof horn growth. The pasture-raised cows of Montbeliarde breed in the measurements of the hooves of the thoracic limbs showed an increase at the end of the experiment: length by 0.6 cm, the width by 0.5 cm, the total width by 0.3 cm, and these measurements for the same barn-raised animals showed an increase: 0.6 cm in length, 0.34 cm in width, 0.25 cm in total width. In pasture-raised Simmental an increase in hoof measurements of the thoracic limbs was observed: length by 0.23 cm, width by 0.21 cm, total width by 0.36 cm. These measurements taken on the same animals reared in a barn showed an increase: length 0.47 cm, width by 0.71 cm, total width by 0.59 cm ($P<0.05$).

The situation with the pasture-raised Montbeliarde breed is as follows: in the measurements of the hooves of the pelvic limbs, an increase was demonstrated at the end of the experiment: length by 0.34 cm, width by 0.21 cm, total width by 0.23 cm, and these measurements, and for the same animals raised in a barn, the following increase is reported: length by 0.47 cm, width by 0.58 cm, total width by 0.25 cm. The measurements of the back hooves of the thoracic limbs of pasture-raised Simmental cows showed an increase at the end of the experiment: the length by 0.52 cm, the width by 0.17 cm, the total width by 0.36 cm, whereas these measurements, for the same barn-raised animals showed an increase: 0.47 cm in stature, 0.59 cm in width, 0.59 cm in total width ($P<0.05$)(Figures 1-4).

The intensity of growth of the hoof horn depends on various external and internal factors, such as breed, sex, technology, sufficiency and dietetics of feeding, season of the year, physiological state, etc. (Cook et al., 2009; Kvochko et al., 2010; Shearer et al., 2013; Zemlyanukhina, 2016; Lomonov & Skorkina, 2020).

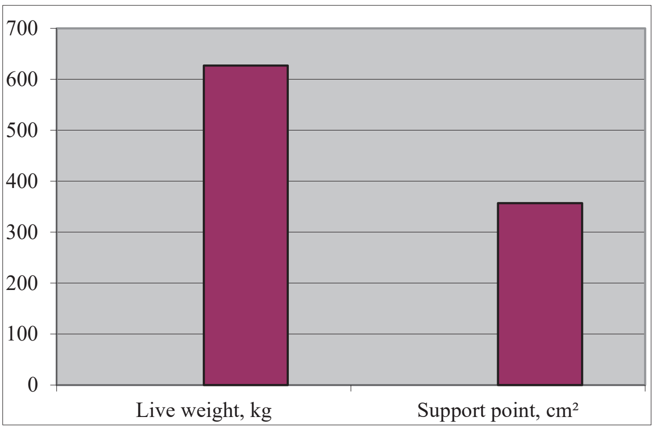

Figure 1. Pasture-raised Montbeliarde

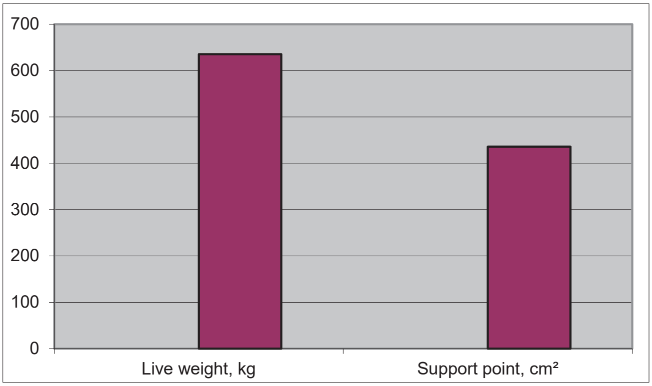

Figure 2. Barn-raised Montbeliarde

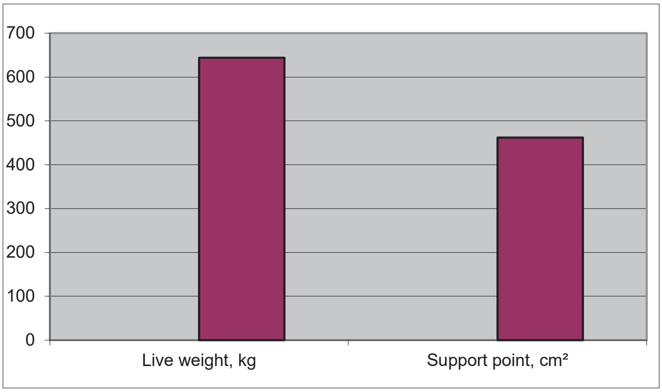

Figure 3. Pasture-raised Simmental

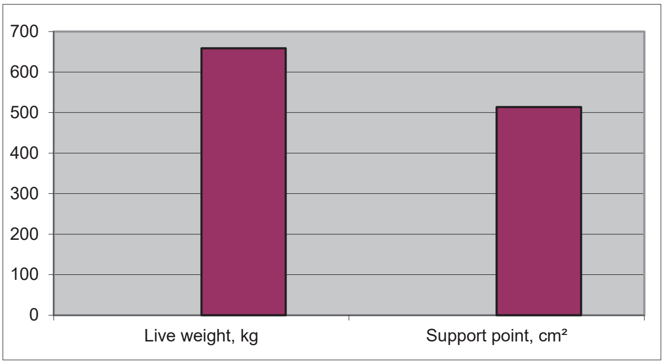

Figure 4. Barn-raised Simmental

CONCLUSIONS

Hoof height varies from 6.3 to 6.9 cm in Montbeliarde cows and 6.5 to 6.9 cm in Simmental cows, and the notched angle in both breeds shows acceptable values of 44˚ to 45˚ for thoracic limbs and 54˚ 56˚ for pelvic limbs ($P<0.05$).

The visual assessment, measurements and calculations of the support point of both groups of examined cows give us a description of their limbs and hooves (Veremei et al., 2010). They showed that in pasture-raised cows in both breeds, a higher coefficient (ratio) per unit of support point, relative to live weight (Table 2), was observed, respectively 1.7576 points for the

Montbeliarde breed or by 8.2% and 1.4869 points for the Simmental breed or by 9.1%. This is explained by the fact that pasture-raised cows show more hoof horn attrition.

The present results are similar to the results obtained by Homin et al. (2017), Longova et al. (2020) and Lemonov & Skorkina (2020), and in some cases complement them.

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STUDY ON THE TRENDS OF MILK PRODUCTION AND DAIRY PRODUCTS AT EUROPEAN AND NATIONAL LEVEL

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Abstract

Production of milk is one of the most problematic sectors of agriculture, both in the EU and in Romania. This paper aims to analyse the evolution of milk production and dairy products at the national and EU level, between 2017 and 2022. The data of the National Institute of Statistics, Eurostat and other public sources were statistically processed for establishing trends at the level of main European milk producers and processors and several countries neighbouring Romania. At the national level, the average quantities of milk collected by the processing units increased slightly from 2017 to 2021, but in 2020 and 2021, the amount of milk has a downward trend. In the first part of 2022, the milk production remained at a low level, only during September-November were higher values recorded than in the same periods of 2020 and 2021. Romania's milk production falls for the second year in a row in 2022, under pressure from rising costs (especially fuel and energy) of the drought that has affected the feed quantity and quality and imposition of prices by large processors.

Key words: *dairy products, European Union, milk production, Romania, trend.*

INTRODUCTION

Global population growth, increasing pressure on natural resources and global warming, determine a new working context at the national and international level. The increase in world demand for food, the increased degree of urbanization, increasing prices for inputs, the high pressure exercised on water resources and the increase in the vulnerability of crops and animals to climate changes will limit food production. It is predicted that, globally, the demand for food will increase by 70% by 2050, as a result of the increasingly large population. All these aspects will have profound implications on agriculture and rural areas. (FAO, 2011).
Agriculture is a field with considerable potential, occupying, by tradition, an important place in the structure of the Romanian economy. Along with the vegetable sector, the livestock sector represents an important branch of the national economy in general and agriculture in particular, providing raw material for the food industry and food for the population.
In order to support the growth of the global population, the dairy industry must look to the future and the specialists must cooperate in terms of obtaining better dairy animals and more productive and sustainable dairy farms. This can be achieved using knowledge and technology from fields such as genomics, microbiomics and intelligent dairy farming systems. Future milk production is predicted to reflect sustainable intensification that benefits animals, agro-ecosystems and humanity by producing key nutrients for human consumption (Britt et al., 2018).
The production of milk and dairy products is one of the most problematic sectors of agriculture, both at the EU level and at the national level. Milk and dairy products are important sources of protein, iodine, calcium and several other vitamins and a daily intake is of high importance for many Europeans. To be named a dairy product, food must be produced from the milk of cows, goats, sheep or similar animals. The dairy sector includes food such as

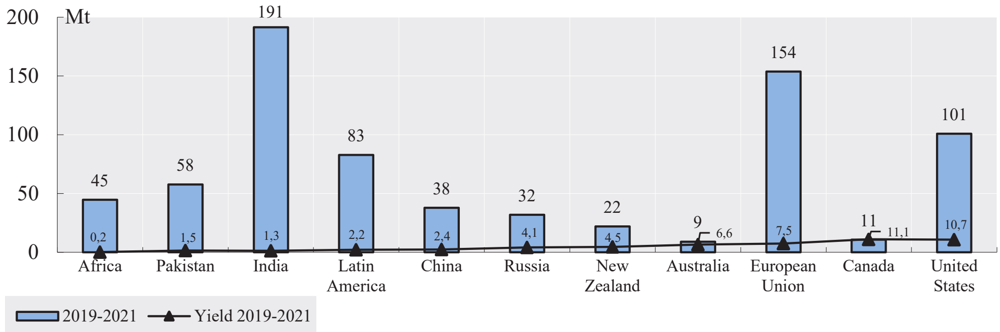
milk, milk powder, cheese, butter and yogurt as well as ice cream (Key et al., 2022).
Milk is a very important food product due to its complex chemical composition, biological value and high digestibility. It contains more than 100 substances needed by the human body: all 20 amino acids, 10 fatty acids, 25 vitamins and 45 mineral elements. This is a product of great socio-economic importance, essential for the physical and intellectual development of the individuals, as well as for maintaining the health of the population (Mihai et al., 2019).
A structural process of transformation is being registered worldwide, as a result of new global challenges with a long-term effect, which require the development of a strategic vision in the field of agriculture.

MATERIALS AND METHODS

Milk and milk product statistics are collected under Decision 97/80/EC implementing Directive 96/16/EC. They cover farm production and utilization of milk (annual), collection (monthly for cows' milk) and production activity by dairies (annual) and statistics on the structure of dairies (every third year). An annual methodological report is also collected (EuroStat, 2023).
This paper aims to analyze, at the national and European Union (EU) level, the production of milk and dairy products in the period 2017-2022. The data used in this paper represent statistical information presented by specialized national, European or global institutions. All data collected from Eurostat and National Institute of Statistics databases was studied and rephrased into the tables and figures. There have been used data series regarding livestock, milk production and dairy products in Romania and EU. The purpose of the statistics is to show changes in the size and value of livestock, total production of milk and milk products.
At the European level, the productions were compared with the big milk producers and processors (Germany, France, Holland, Poland and Italy) and with several countries neighboring Romania (Bulgaria, Serbia and Slovakia).

RESULTS AND DISCUSIONS

Milk and dairy products are vital sources of nutrition and provide livelihoods for millions of people in the dairy value chain across the world. World milk production (roughly 81% cow milk, 15% buffalo milk, and 4% for goat, sheep and camel milk combined) increased by 1.1% to about 887 Mt in 2021, primarily driven by an expansion in output in India and Pakistan due to a continued increase in dairy herd numbers and fodder availability helped by favorable monsoon rains. Milk production in the three major dairy exporters, New Zealand, the United States, and the European Union varied from a marginal to modest increase to a slight decline, respectively. Milk is produced on a large scale in the European Union, contributing around 20.69% to total world production (OECD-FAO, 2021). Under these circumstances, the EU is a major player on the world milk market, as can be seen in Figure 1.



Note: The yield is calculated per milking animal (mainly cows but also buffaloes, camels, sheep and goats)

Figure 1 Milk production and yield in selected countries and regions of the world (OECD-FAO, 2021)

The EU dairy animal herd (cows, goats, and sheep) has declined in recent years as milk yields per animal have improved. In 2020, there were approximately 20 million cows in the EU, each producing an average of 7,300 kg of milk. It is expected that the number of animals will continue to decrease and dairy cow numbers in the European Union are forecast to fall below 20 million head in 2023, a decline of 1.7 million head since its peak in 2016, and a decrease of 564,000 head since 2021 (Table 1, Figures 2-4).

Table 1. Dynamics of livestock for dairy animals, in UE (thousand heads)

Year	**Animal category**	**Country**								
		Slovakia	**Bulgaria**	**Serbia**	**Romania**	**Netherlands**	**Italy**	**Poland**	**France**	**Germany**
2017	dairy cows	129.86	260.78	429	1175.4	1665	2040.11	2152.9	3596.84	4199.01
	goats	37.07	256.97	183	1583.3	546	992.08	n	1213	140
	sheep	365.34	1316.78	1704	9981.8	1015	7215.4	n	6877	1579.79
2018	dairy cows	127.87	244.36	423	1158.2	1552	1939.48	2214.1	3554.23	4100.86
	goats	36.91	271.74	196	1539.3	518	986.26	n	1252	146
	sheep	351.12	1350.03	1712	10176.4	743	7179.12	n	7166	1569.9
2019	dairy cows	125.85	226.69	423	1138.8	1590	1875.72	2166.9	3490.81	4011.67
	goats	35.59	228.49	191	1594.8	551	1058.72	49.9	1242	141
	sheep	320.56	1280.98	1642	10358.7	758	7000.85	268.54	7105	1556.5
2020	dairy cows	122.05	241.94	417	1121.9	1569	1871.27	2125.7	3405.68	3921.41
	goats	n	253.4	202	1611.8	557	1065.71	n	1413.98	161
	sheep	n	1307.77	1685	10281.5	710	7034.16	n	6998.71	1483.7
2021	dairy cows	120.07	230.34	408	1081.9	1554	1844.37	2035.2	3322.03	3832.72
	goats	n	215	195	1492.5	575	1060.75	n	1387.77	164
	sheep	n	1199.55	1695	10087.4	729	6728.35	n	6994.63	1508.1
2022	dairy cows	115.95	212.53	374.2	1080.8	1570	1865	2037.28	3230.86	3809.72
	goats	n	184.74	191.7	1504.7	570	1010	n	1310.71	159
	sheep	n	1089.7	1720.83	10442.9	724	6568	n	6597.52	1507.5

n - not available data
(Eurostat, 2023, TAG00014)

In terms dairy cows number, Romania ranks 4th among the compared EU countries, but in terms of the number of goats and sheep, Romania is in first place and the number of sheep even registering an increase in 2022, compared to all previous years.

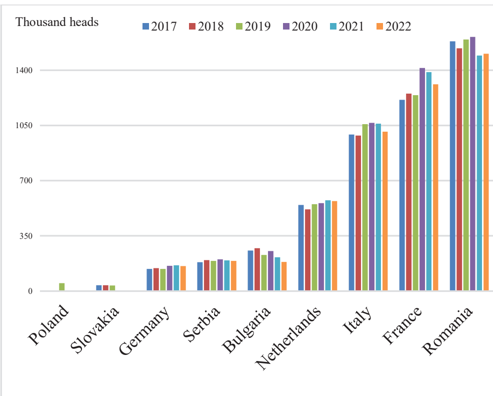


Figure 2. Number of dairy cows in UE

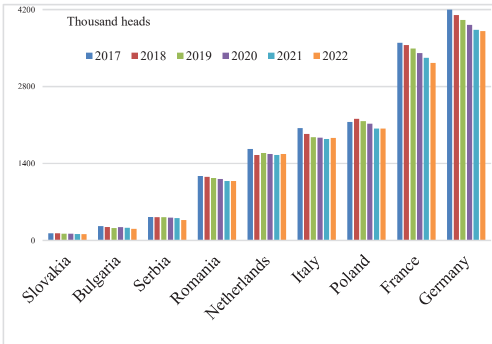


Figure 3. Number of goats in UE

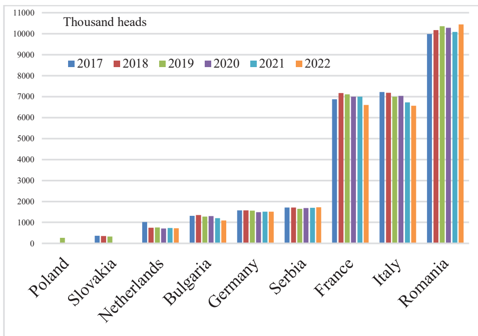


Figure 4. Number of sheep in UE

Milk production on the farm covers milk from cows, ewes, goats and buffaloes (Table 2, Figure 5).

Table 2. Production of milk on farms, in UE (1000 t)

Year / Country	2017	2018	2019	2020	2021
Slovakia	923.40	917.00	915.70	929.54	914.41
Bulgaria	1 091.47	1 025.20	939.50	1 005.45	953.07
Serbia	1 599.26	1 590.01	1 597.04	1 583.74	1 563.48
Romania	4 439.20	4 443.30	4 339.60	4 362.50	4 299.70
Italy	12 983.23	13 131.64	13 300.10	13 509.51	13 997.97
Poland	13 702.38	14 179.21	14 511.49	14 830.87	14 890.27
Netherlands	14 822.00	14 426.00	14 944.00	14 932.00	14 607.88
France	26 006.31	26 022.50	26 036.29	26 288.53	25 834.80
Germany	32 614.17	33 109.66	33 102.57	33 188.89	32 531.56

(EuroStat, 2023)

At the EU level, Germany produces the largest amount of milk, at the farm level, closely followed by France.

The Netherlands, a large producer of milk as well, is on the 3rd place in this top, with a production of approx. 50% of that of Germany.

In Romania, milk production is decreasing in the 5 years presented, reaching 4299700 tons in 2021, i.e. about 12.5% of Germany's production.

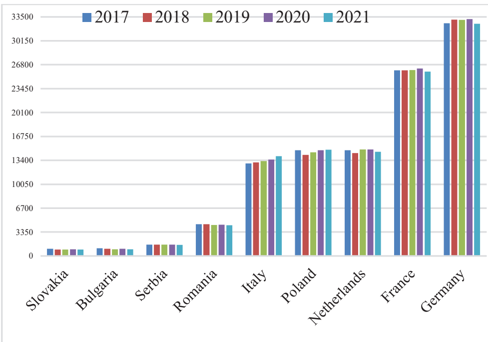


Figure 5. Dynamics of total milk production on farms, in UE (1000 t)

Figure 6 shows the value of cow milk production in the European Union from 2017 to 2022. In 2022, the EU cow milk production was approximately 143.9 million metric tons, decreasing from about 157.5 million tons in 2020, when it had a maximum in the 5 years analyzed.

The trend of such a steep decrease at the EU level can be attributed to the fact that United Kingdom cow's milk production is no longer taken into account (Shahbandeh, 2023)

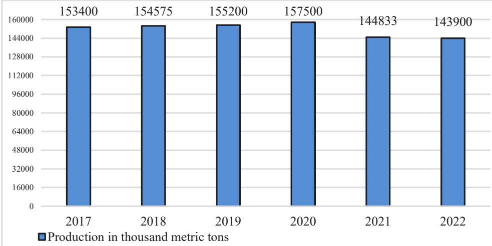


Figure 6. Cow milk production in the EU, between 2017-2022

Cow milk production (1000 t) in the under discussion EU countries is shown in the Table 3 and Figure 7.

Table 3. Production of cow's milk on farms, in UE

Year / Country	2017	2018	2019	2020	2021
Bulgaria	968.00	898.77	822.00	882.00	836.00
Slovakia	911.73	904.62	904.26	917.69	902.64
Serbia	1 550.70	1 537.38	1 553.84	1 539.45	1 517.69
Romania	3 797.70	3 797.60	3 663.20	3 679.60	3 637.00
Italy	12 198.88	12 339.75	12 494.40	12 712.48	13 202.45
Netherlands	14 501.00	14 090.00	14 555.00	14 522.00	14 217.00
Poland	13 694.00	14 171.00	14 503.00	14 503.00	14 881.00
France	25 055.20 (p	25 055.10 (p)	25 062.00	25 234.84	24 778.84
Germany	32 598.20	33 086.81	33 080.18	33 164.91	32 506.91

(EuroStat, 2023)

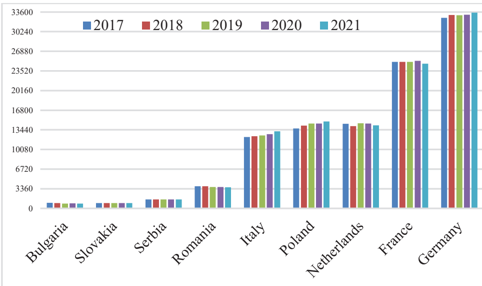


Figure 7. Dynamics of cow's milk production on farms, in UE (1000 t)

Cow's milk production has a linear trend in Slovakia, Serbia and the Netherlands, slight decreases are recorded in Bulgaria, Romania and France, and increases in production are recorded in Italy, Poland and Germany.

In Table 4, Figure 8, data covers cow's milk collected (1000 t) in UE, in farms, by approved dairies. A distinction should be made between "milk collected by dairies" and "milk production on the farm". Milk collection is only a part of the total use of milk production on the farm. The other part of the use of milk produced on the farm generally includes domestic consumption, direct sale and cattle feed (Eurostat, 2023; INSS, 2017-2022)

Table 4. The quantities of cow's milk collected by processing units, in UE

Year / Country	2017	2018	2019	2020	2021
Bulgaria	592.49	648.80	658.77	694.18	679.06
Slovakia	825.89	818.22	814.73	833.79	822.96
Serbia	862.07	866.97	873.95	907.65	891.80
Romania	1 028.33	1 109.31 (p)	1 122.33 (p)	1 134.90 (p)	1 125.66 (p)
Poland	11 647.09	11 945.62	12 174.96	12 457.39	12 515.42
Italy	11 902.24	12 070.75 (p)	11 965.01 (p)	11 894.79 (p)	13 042.03
Netherlands	14 295.90	13 880.80	13 801.90	13 986.60	13 603.40
France	24 629.49 (p)	24 542.54 (p)	24 526.30 (p)	24 602.21 (p)	24 198.75 (p)
Germany	31 937.03	32 490.94	32 442.21	32 548.98	31 942.32

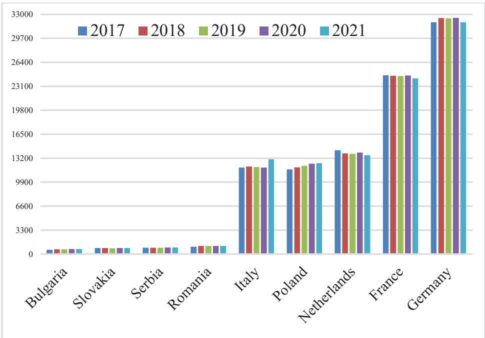


Figure 8. Dynamics of cow's milk collected, during 2017-2022, in EU (1000 t)

Correlated with the evolution of cow's milk production, it is to a certain extent the collection of milk by the processing units. In countries where the evolution of cow's milk production has a linear aspect (Slovakia and Serbia), the linearity of its collection is also observed. Also, where there is an increase in production, there is also an increase in the amount of collected milk (Italy, Poland). Although the production of cow's milk was linear, in the Netherlands or even increasing in Germany, in these countries there is a tendency to decrease the collection of milk. In Romania, although the production of cow's milk is decreasing, the collection it has an increasing trend.

For a better observation of the evolution of the amount of milk collected (1000 t) by the processing units in Romania, for the 2017-2022 period, the data from the National Institute of Statistics, were used (Table 5 and Figure 9).

In 2018 compared to 2017, the amount of cow's milk collected by processing units increased by 81,941 tons (+8.0%). In 2019 compared to 2018, the amount of cow's milk collected by processing units increased by 12556 tons (+1.1%). In 2020, compared to 2019, the amount of cow's milk collected by processing units increased by 12,572 tons (+1.1%). In the period 2021 compared to the period 2020, the amount of cow's milk collected by the processing units decreased by 9238 tons (-0.8%). In the period 2022 compared to the period 2021, the amount of cow's milk collected by processing units increased by 8138 tons (+0.7%).

Table 5. The quantities of cow's milk collected between 2017-2022, in Romania

Period	2017	2018	2019	2020	2021	2022
UM	tons					
January	72819	81986	86720	85641	87405	85123
February	71081	76835	82336	85122	81083	79120
March	85207	89502	98315	95737	95704	91187
April	85103	94161	98912	94834	96117	90770
May	103657	111428	111765	107611	113342	106880
June	102440	105017	104156	108979	111706	105594
July	97295	106679	103947	106965	104863	99341
August	89851	98552	94953	99082	95660	97837
September	83982	88802	87975	91522	89092	94464
October	83246	89843	87255	89988	88129	98828
November	75272	82368	81349	83537	80437	92023
December	78381	84597	84644	85881	82124	92631
Annual average	85694.5	92480.83	93527.25	94574.92	93805.17	94483.17
Annual total	1028334	1109770	1122327	1134899	1125662	1133798

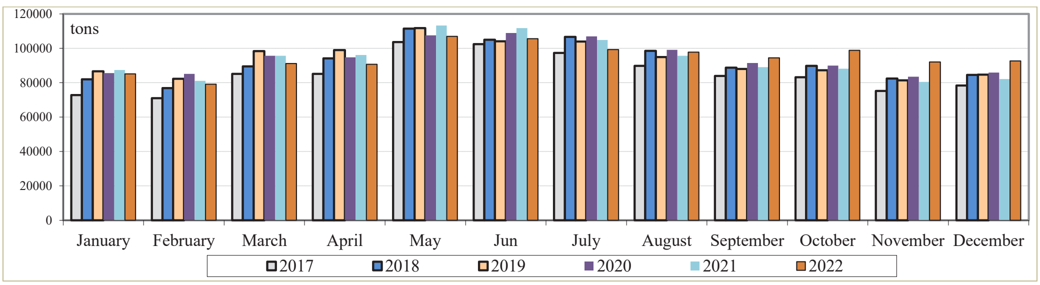


Figure 9. Dynamics of cow's milk collected, during 2017-2022, in Romania

In Romania, the average quantities of milk collected by the processing units increased slightly from 2017 to 2021 from 85694.5 to 93805.17 (8.64 %).

Even if compared to the years 2017, 2018 and 2019 in 2020 and 2021 there is an increase in milk production, the amount of milk produced has a downward trend. In the first part of 2022, the amount of milk remained at a low level, only in September, October and November registering higher values than in the same periods of 2020 and 2021.

There was made an analysis of the quantities of some dairy products (cheese, butter, drinking milk and cream) obtained in EU, in the period 2017-2022. No data were available regarding the production of drinking milk and cream for the Netherlands.

As expected, the large milk-producing countries also have the largest production of dairy products. Thus, Italy, Poland, France and Germany have the largest quantities of cheese, butter and cream. Also, at the level of these countries, the largest quantities of milk are consumed (Tables 6-9, Figures 10-13).

Several cheese types belong to database named "cheese". Data presented in Table 6 relate to all seven cheese types with different moisture contents and compositions (EuroStat, 2023).

Table 6. Production of cheese (1000 t)

Year / Country	2017	2018	2019	2020	2021
Slovakia	40.31	42.72	43.30	43.29	44.34
Serbia	50.09	53.10	n	53.37	53.30
Romania	91.07	96.24	96.40	97.36	98.82
Bulgaria	89.40	91.90	99.95	103.39	101.46
Poland	840.63	855.59	867.95	893.78	919.71
Netherlands	896.00	902.00	953.26	999.60	984.05
Italy	1 261.13	1 308.03	1 327.30	1 344.69	1 374.23
France	1 919.57	1 907.76	1 903.29	1 862.10	1 865.96
Germany	2 216.55	2 245.80	2 297.40	2 355.12	2 360.90

n - not available data (EuroStat, 2023, TAG00040).

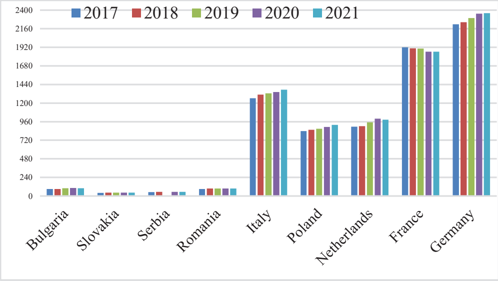


Figure 10. The dynamics of cheese production in the EU (1000 t)

Slovakia, Serbia and Romania showed a small increase in cheese production in the analyzed years, while Bulgaria (+10%), Poland, Italy, Holland and Germany (+6.5%) showed obvious increases. France is the only country observed, which showed a decrease of approximately 1%.

Data concern the total production of butter and other yellow fat dairy products are presented in Table 7 and Figure 11.

Table 7. Production of butter, in EU (1000 t)

Year / Country	2017	2018	2019	2020	2021
Bulgaria	1.06	1.07	1.09	1.03	1.26
Serbia	3.97	4.17	n	5.35	5.28
Romania	12.11	10.87	8.86	10.22	9.48
Slovakia	n	10.12	10.06	11.08	10.72
Italy	91.22	97.48	94.03	92.25	94.15
Netherlands	248.00	247.00	229.53	220.71	201.86
Poland	213.72	222.66	224.45	243.39	232.17
France	412.72	417.41	419.22	417.54	410.54
Germany	488.11	474.88	490.65	497.30	461.68

n - not available data (EuroStat, 2023, TAG00038)

For the production of butter and dairy products based on yellow fat, Bulgaria have very low values, and, together with Slovakia they show no changes throughout the 5 years analyzed.

France (-2.14%) and Germany (-5%) showed reductions in butter production. The Netherlands and Romania had significant reductions of 19% and 21% respectively.

The only countries taken into account that showed an increase in butter production were Serbia (+8.5%), Italy (+3.2%) and Poland (+33%).

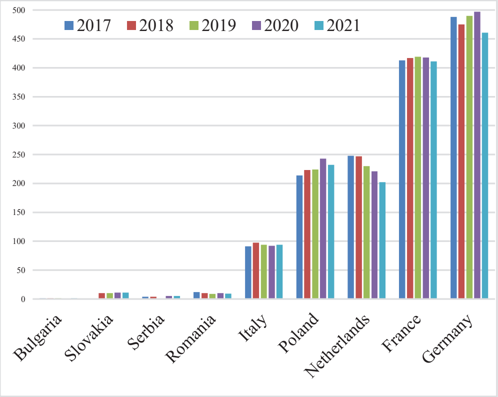


Figure 11. Dynamics of butter production, in EU (1000 t)

Data concerning drinking milk production are presented in Table 8 and Figure 12. Decreases in drinking milk production are observed in Serbia, Slovakia, France and Germany. Bulgaria, Romania and Poland show increases in this production.

Table 8. Drinking milk production, in EU (1000 t)

Year / Country	2017	2018	2019	2020	2021
Bulgaria	66.11	74.97	74.90	76.11	77.28
Serbia	225.27	228.90	209.73	234.68	208.97
Slovakia	248.43	242.92	227.58	255.18	243.94
Romania	289.96	306.22	331.34	358.07	387.21
Poland	1 732.69	1 776.80	1 891.90	1 986.72	1 958.32
Italy	2 459.03	2 469.58	2 298.86	2 313.83	2 481.46
France	3 229.51	3 102.61	3 007.98	3 067.96	2 802.19
Germany	4 743.28	4 646.12	4 522.96	4 566.07	4 379.69

(Eurostat, 2023, APRO_MK_COLA)

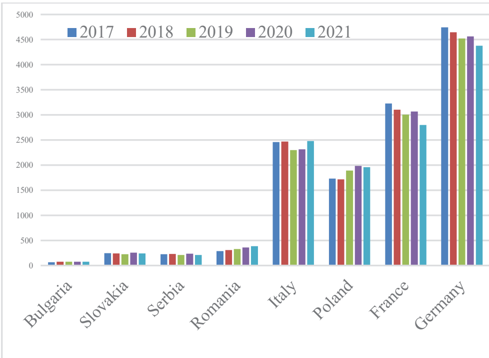


Figure 12. Dynamics of drinking milk production, in EU (1000 t)

In Table 9 and Figure 13 are presented the data related to the production of cream.

Table 9. Cream for direct consumption (1000 t)

Year / Country	2017	2018	2019	2020	2021
Bulgaria	2.34	2.76	2.79	2.87	3.80
Slovakia	30.46	28.38	27.72	29.63	32.06
Serbia	28.21	27.42	29.04	32.37	32.86
Romania	66.46	66.43	68.12	68.32	67.75
Italy	132.12	141.48	136.12	135.75	149.83
Poland	264.80	262.15	254.52	259.43	259.89
Germany	577.99	552.21	554.41	529.98	544.39
France	502.89	503.07	514.13	535.13	547.67

(EuroStat, 2023, APRO_MK_COLA)

Increases in the amount of cream produced in the EU were registered in all the countries observed, with the exception of Germany, which recorded a decrease of 5.71%, thus being surpassed by France, whose production increased by about 9%.

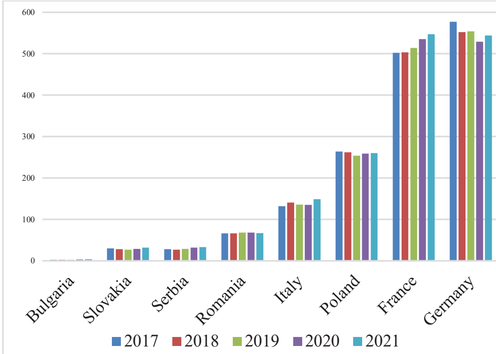


Figure 13. Dynamics of cream production, in EU (1000 t)

In order to be able to discuss the 2017-2022 evolution of the production of dairy products in Romania, there were used the data provided by National Institute of Statistics (Table 10).

Table 10. Production of dairy products, in Romania, in 2017-2022 (tons)

Year	Drinking milk	Cream for consumtion	Butter	Cheese
2017	76489	15880	3221	20437
2018	306221	66887	10881	94285
2019	331344	68114	10653	96717
2020	358072	68320	12163	97292
2021	387208	67751	11198	98514
2022	370120	64978	10483	101972

Data on dairy production values collected from INSS may differ slightly from Eurostat databases, due to monthly/annual data revision and rounding.

In 2018, compared to 2017, there were increases of 17,248 tons of drinking milk production (+6.0%) and 3,214 tons of cheeses (+3.5%). The production of butter decreased by 1226 tons (-10.1%), and the consumption of cream remained approximately the same as in 2017.

In 2019, compared to 2018, increases in production were recorded in: drinking milk with 25,123 tons (+8.2%), cheeses with 2,432 tons (+2.6%) and cream for consumption with 1,227 tons (+1, 8%). Butter production decreased by 228 tons (-2.1%).

In 2020, compared to 2019, there were increases in the production of all dairy products under observation. Thus, for butter the increase was by 1510 tons (+14.2%), for drinking milk with 26728 tons (+8.1%), for cheeses with 575 tons (+0.6%) and for cream with 206 tons (+0.3%)

In 2021, production decreased in butter by 965 tons (-7.9%) and in cream by 569 tons (-0.8%). However, increases in production were recorded for drinking milk by 29,136 tons (+8.1%) and cheeses by 1,222 tons (+1.3%).

Cheese production increased in 2022 by 3458 tons (+3.5%). There were decreases in butter production by 715 tons (-6.4%), drinking milk by 17088 tons (-4.4%) and cream for consumption by 2773 tons (-4.1%).

CONCLUSIONS

The European Union is a major producer of milk and milk products as part of the common market organization (CMO). Milk production takes place in all EU countries and represents a significant proportion of the value of EU agricultural production. In the EU, total milk production is estimated at around 155 million tons per year. The main producers are Germany, France, Poland, the Netherlands, Italy and Ireland. Together, they account for almost 70% of EU milk production.

Dairy cow numbers vary enormously between European countries, as do yields. However, as the dairy sector develops across the EU, yield variations and technical disparities have narrowed. Less advanced manufacturers are quickly catching up with those who restructured and modernized before them.

Together with the decrease in the herds of cows and goats in Romania, the total amount of milk produced by dairy animals also decreased, the increase in herds of sheep (by 4.61%) failing to compensate for this decrease.

Romania's milk production falls for the second year in a row in 2022, also under pressure from rising costs (especially fuel and energy) of the drought that has affected the quantity and quality of feed and the pressure of large processors to impose prices.

However, the quantities of milk collected by milk processing factories had an increasing trend. This also led to the manifestation of a general tendency of increasing in the production of dairy products.

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THE STAGE OF RESEARCH ON WELFARE REQUIREMENTS IN LIVESTOCK FARMS

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Abstract

In order for animals to express their genetic potential and their production not to be affected, their welfare is a must. This study analyzed the origins of the concept of welfare and what animal welfare means, which implies the 5 freedoms that must be respected. Then, it was briefly analyzed what the consumer`s consent paying a higher price for products obtained from farms that applied friendly animal technologies means. After that, it was analyzed the most important aspects that the farmer faces and can affect the animals welfare. At the end, the interaction of man-animal was brought into account, which has an enormous importance for animals welfare.

Key words: *animals welfare, farm welfare, farm, welfare.*

INTRODUCTION

For studying and understand the concept of animals welfare, we have to analyze the breeding systems, the legislation for farm animals and here it should be mentioned that it is different from country to country. Another important aspect is the production costs.

Natural resources and feeding stuffs and work costs are the primary elements of competitiveness in the farm animal and meat processing areas (Van Horne & Achterbosch, 2008; Cziszter et al., 2010).

Systems that deal with animal welfare are:

1) Welfare Quality® protocol;
2) ANI 35L.

It cannot be said that they are 100% complete and that by applying and observing them strictly, the animal welfare in conventional or organic farms has been solved.

However, not all factors can be included in legislative regulations and even for those that are included, there is usually a range in which farmers can fit in. So, due to the combinations of factors that vary between certain limits, a very large number of different situations are reached. That’s why, it’s essential to be identified the most important elements and narrow their interval as much as possible (Venglovsky, 2010).

A final aspect to be mentioned and of major importance, is the interaction of animal man. In this interaction, so much in the farm (paddock, hall, milking parlour, etc.) but also at slaughter, must be minimized and if it can even be excluded, the fear induced by the caregiver to the animal.

MATERIALS AND METHODS

The researches with reference to the notion of the concept of animal welfare were realize by analyzing scientific articles, publications from the European Union Commission, specialized book on the relationship between animal welfare and quality of the resulting products.

Besides the fact that the origin and concern for the concept of animal welfare was shown, it was generally followed, the implications of this concept within conventional and organic farms, with the help of welfare quality and ANI 35L systems, the major problems in farms, the legislation in force, but also some differences in terms of the price of the finished product.

It has been shown in short, the impact of human animal interaction, which can have negative effects and is undesirable. And the positive impact that is desirable must necessarily be manifested also by the suppression of the induction of fear by caregivers in animals.

RESULTS AND DISCUSSIONS

To understand animal welfare, we must look back in time and to analyze the debates and concerns that made welfare in zootechnical farms a problem that needed to be solved but in the same time the farm to remain profitable.
The first concern and criticism of livestock internment systems was presented in the book "Animal Machines", from animal defender (Harrison, 1964; Fraser, 2008) and described cages for laying hens and crates for veal calves, and she claimed that these systems are so unnatural that they cause animals to lead miserable and unhealthy lives.
On "Animal Liberation", australian philosopher (Singer, 1975; Fraser, 2008) based his critical remarks of confinement production on the principle that actions should be judged right or wrong on the basis of the pain or pleasure that they cause.
Into those and other quotations a crucial worry focus on words as "pleasure", "pain", "suffering", and "happiness" (Fraser, 2008).
A British committee, that was formed to classify the farm animals welfare determined: „In principle we find unacceptable of a degree of confinement of an animal which necessarily frustrates almost all the activities which create his natural behaviour" (Brambell, 1965; Fraser, 2008).
In Sweden Astrid (1989) and Fraser (2008) say: "Let farm animals see the sun just once, let them get to breathe fresh air for once, instead of manure gas".
In USA the philosopher (Rollin, 1993; Fraser, 2008) made this statement: Welfare not only represent control of pain and suffering, also will entail nurturing and fulfillment of the animal naturalness.
If we watch closely, welfare concept comes from different persons but not from farmers and without the right of reply the confinement system it can be perceived as very cruel. To make an idea about animal welfare, we need to see other opinions: "My experience was that by and large the standard of welfare amid animals kept in the so called "intensive" systems is higher" (Taylor, 1972; Fraser, 2008). Also, Sainsbury (1986) and Fraser (2008) expresses himself in this way: Good health is the birth right of each animal that we rear, whether intensively or under other conditions.
A good definition for animal welfare is giving by this fundamentals: The animal normal biological function (which, amid other things, means assuring that the animal is healthy and properly nourished), his emotional state (inclusive of the nonappearance of negative emotions, suchlike pain and chronic fear), and his capability to express certain normal behaviors (Fraser et al., 1997; Manteca et al., 2012). Also, in 1997 the welfare of intensively kept pigs was reviewed from the scientific veterinary committee, EU, Brussels (Fraser, 2008) and finded: Several serious welfare problems for sows persist even in the best stall housing system.
The review effect was that European Union approved a regulation to interdict the gestation stall starting in 2013 (Fraser, 2008).
As is mentioned in the speciality literature from 1965, the concept of five freedoms become a necessity (Szücs & Cziszter, 2010a).
The "Five Freedoms" principle provide a very helpful and practical approach to examine welfare, specifically, to its assessment in livestock farms, during the transport and slaughtering farm animals (Manteca et al., 2012).
The five freedoms are (Manteca et al., 2012):

1) The animal is free from hunger, thirst and malnutrition.
2) The animal is free from physical and thermal discomfort.
3) The animal is free from pain, injury and disease.
4) The animal is able to express most of its normal behavioral patterns.
5) The animal does not experience fear or distress.

Those five freedoms mentioned above, are not something to be 100% complete and is more for animal protection in his/her productive life.
Even so, regardless of its clear helpfulness, it has two deficiencies, first, it is sometimes too general and second, there is a certain overlapping between some of the five freedoms (Manteca et al., 2012).
A solution for stoping the debate on welfare for animals, was and still is Welfare Quality® protocol, founded by the EU.

Different from other protocols, which predominantly applies environmentbased parameters, the protocols of the Welfare Quality® project are mainly established on animal based measures (Manteca et al., 2012).
In conformity with Welfare Quality® protocols, animal welfare assessments need to respond at four questions (Manteca et al., 2012):

1) Animals are properly fed?
2) Animals are properly housed?
3) Animals are healthy?
4) Animals behavior reflect optimised emotional states?

We live in a world where we need to integrate the animal products but from animal welfare perspective. There is a new notion in economy and that notion is *circular welfare economy* (CWE) and is first time presented by Bracke (2017). *An essential element of sustainable, circular farming: Integrity & a circular welfare economy. Retrieved from http://marcbracke.nl/ an-essential-element-of-sustainable-circular-farming-integrity-a-circular-welfare-economy/.*
The idea of a CWE was to accentuate that in a transition in the direction of circular agriculture we should not forget about animal welfare (Bracke et al., 2022).
Meaning that we're supposed to not respect animal welfare only because some people find it necessary, but because it is a purpose in itself (Bracke et al., 2022).
As I mentioned earlier the purpose of Welfare Quality® project was to provide science-based practical tools and strategies to improve the well-being of animal farms (Szücs & Czisztér, 2010b). The foundations of the project put into practice three ways to improve the well-being of farm animals, as shown in Figure 1 (Blokhuis, 2004) and Figure 2 (Blokhuis et al., 2003; Szücs & Czisztér, 2010b).
We may think and believe that is enough and by respecting the Welfare Quality® project the animal welfare issues have been resolved, but it is not so.
The next factors, presented in descenting order, seem to be very necessary for welfare and protection of animals (Martelli, 2009; Czisztér et al., 2010): space allocation, friendly transport, access to outside areas, natural light exposure, nonappearance of movement restrictions by tying with chains either ropes, natural behaviors expression, nonappearance of social contact and mutilation.

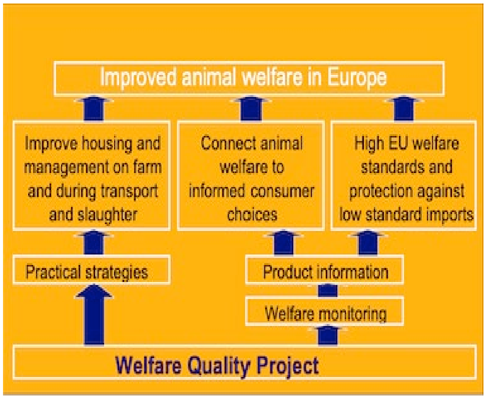

Figure 1. Directions of the Welfare Quality® Project to satisfy animale welfare (Blokhuis, 2004)

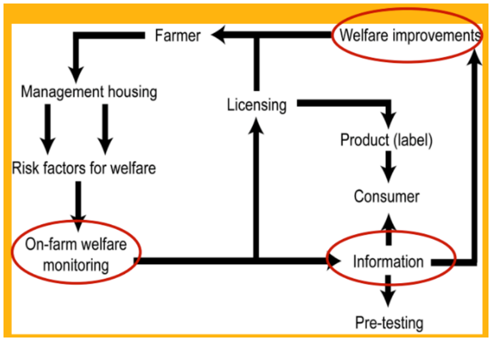

Figure 2. Schematic representation of the roles of the monitoring systems on the farm (Blokhuis et al., 2003; Szücs & Czisztér, 2010)

The reality in European Union it's another. According to an report for cattle welfare in dairy farms, member states deficiency of national systems to gather and analyze data from farms which would let a robust assessment of dairy cows welfare (DG SANTE, European Commission).
Let's analyze a little some important aspects, to really understand the concept of animal welfare and I mean husbandry technology of animal farm (intensive, semi-intensive and extensive) compared to organic farms and in the end the final product (meat, milk, eggs, etc) because animal products from conventional farms haves a price and from organic farms or conventional farms but apply the animal welfare haves a different price.

Even though farm animals welfare it is a problem that is of increasing concern to european citizens, there is a higher inclination to buy the most inexpensive meat (EC, 2007; Cziszter et al., 2010). Now, because we are in 2023 and price of the energy is higher from 2007 and we need to understand very well why to pay more for an aminal product that's coming from organic farms or farms that apply animal welfare.

Due to selection for high productions, modern breeds of animals may be unable to reach their genetic potential in an organic system (Kovács & Konrád, 2010). Therefore it would be better to use local breeds in organic production that are better adapted from a genetic point of view to their environment (van Diepen et al., 2007; Kovács & Konrád, 2010).

But what about high production of modern breeds farm animals? The second question: What about intensive farms but to apply the animal welfare concept? And questions are many but the consumer is the last to decide and how to send the animal welfare message in the market and the consumer to pay more for it?

These products may be those that are explicitly labeled as being produced in technologies that ensure animal welfare highest level or organic products, as well as those that have a certain geographical origin (Cziszter et al., 2010).

In European Union, eggs are presented to consumers with different standards of animal welfare in the most explicit way. EU legislation provides for three accommodation systems for laying hens (Directive 1999/74/EC):

1) conventional cages; 2) enriched cages and 3) alternative systems (Cziszter et al., 2010). Danish consumers usually agree to pay more for labels indicating animal welfare production methods (Wier et al., 2005). As we can suppose and we can see this in the grocery store, those eggs with the label *ground production in shelter* and *production in freedom* will have a higher price than eggs with the label *production in conventional batteries*.

A comparison of the price of broilers in the UK's largest supermarkets indicated that whole fresh standard chickens can be bought at a price between GBP 1.78 and GBP 2.99 per kg, the price depending on the size of the finished chicken. Free-range chickens are found at prices
ranging from GBP 3.17 to GBP 5.99 per kg (compared to the prices of organic chickens which are found at prices ranging from GBP 4.24 to GBP 6.25 per kg). This shows that the legal wealth price increase is 6% to 250% higher. It's not understandable to what degree welfare is a dilemma when purchasing organic products (McVittie et al., 2006; Cziszter et al., 2010). Here I must mention that the price is also influenced by the transportation and the addition of the store especially in free-range chickens and organic chickens.

Slovak consumers concerns about pork focus not only on safety issues, but also on price availability and eating habits (Bielik & Šajbidorová, 2009). Also in my native country Romania, pork consumption is average during the year but during the celebration of the birth of Jesus Christ, we have the habit of consuming excess pork, but the price differs from year to year.

A recent study that used the evaluation method to determine people's preference for pork produced in different systems in the U.S.A. showed that consumers consent to pay, on average, 1.50 USD for one kg of pork obtained in the cage rearing system, 1.63 USD for pigs raised in boxes, 3.33 USD for pigs raised in open shelters, 3.51 USD for pigs raised on pasture and 3.80 USD for organically produced pigs (Cziszter et al., 2010).

As for beef, the habit of eating white veal and beef meat matters a lot.

The additional prices that consumers in the U.S.A. and Canada have consented to pay for beef steak are shown in Table 1 (Dickinson et al., 2003; Cziszter et al., 2010).

The beef labeling system was adopted and imposed as a compulsory system in Finland in 1998. For consumers this means that the packaging or labeling attached to beef meat is marked with the text "Finnish beef". The study carried out to examine the consent to pay for the additional information on the label showed that 59% of Finnish consumers agreed to pay an additional price to receive this additional information on the beef label (Latvala & Kola, 2004).

Table1. Consent to pay for roast beef in U.S.A and Canada (Dickinson et al., 2003; Cziszter et al., 2010)

Attribute	Additional price (%)	
	U.S.A	Canada
Humane treatment of the animal	16	19
Greater food safety	20	18
Traceability	7	9
All of the above attributes	35	37

For dairy products there is a wide range, from different species, so I will be brief. In a study constituted consent to pay for yogurts made in Italy, (Napolitano et al., 2008; Cziszter et al., 2010) they founded that in each product studied, consumers expressed a higher consent to pay for products whose labels indicated higher standards of welfare in comparison to yogurts that had labels describing intermediary or lower standards of welfare. These results indicated that information reffering to animal welfare, when provided to customers, may become a considerable factor in their acceptance to pay for products of animal origin.

The price difference is also in the livestock system and because is known the difficulties faced by farmers, a strong and convincing message is required on packaging to products labeled from organic farms, free-range, extensive growth system, etc.

Pig production outside the shelter provide to animals an expanded environmental diversification and freedom of expression of behavior, but demand challenges for breed adaptation, management control, biosecurity and environmental protection (Edwards, 2005; Kovács & Konrád, 2010). In the majority conventional production systems, only adult animals and infant pigs are kept on pasture. In traditional systems and organic production systems, animals for meat can live out of the shelter for their entire life. Indirect consequences can outcome from both positive and negative impacts, the response to physiological stress before slaughter (Kovács & Konrád, 2010). When we are aware of stressors, it is known that porcine stress syndrome is the main cause of deadly under the influence of stressful conditions.

In extensive systems, animals may have the opportunity to express their natural behaviors, but environmental control is more difficult to achieve. Intensive systems restrict the natural behavior of animals which leads to behavioral problems and aggression even if there is better environmental control and easier access to animals to control an individual (Kovács & Konrád, 2010).

In Sweden, a comprehensive research program for organic pig production in stalls with and without access to pasture was initiated to develop a sustainable and efficient pig production system for meat with a top level about animal welfare. Some of the investigations were conducted by (Olsson et al., 2007; Kovács & Konrád, 2010), with a focus on the accommodation systems in which the animal welfare, production, straw use, health status, labor needs, environmental aspects, the use of nutrients by plants and environmental pollution in connection with the losses of N, P and K were taken into account, as well as the degradation of the pasture.

As for sheep, it cannot be said exactly which system is better, conventional or organic because their growth is mostly carried out in a extensive system.

In birds, however, the differences are enormous in terms of conventional farming system and organic farming system.

In poultry, comparing organic poultry farms with conventional ones (Castellini et al., 2006; Kovács & Konrád, 2010), he concluded that all energy indicators are favorable to the organic system, especially regarding:

- High efficiency in turning inputs into the end product;
- High level to removable inputs;
- High level to local inputs;
- Low flow density of energy, materials.

The performances of the two growth systems have big differences in terms of terminal weight, slaughter age, specific consumption and mortality. Conventional systems by using fast growing genotypes bred in very well managed environments and because of veterinary treatments are able to reach heavier animals in 49 days. Organic chickens must remain on the farm for 81 days without any additional prophylactic measures. Thus, only certain growth models maximize all the potential benefits of organic agriculture. Meaning in addition to productivity and profitability and the interactions between all the factors to be taken into account, such as the welfare and intrinsic quality of the meat in the production of organic meat (Kovács & Konrád, 2010).

In husbandry systems on freedom in large flocks, inclusive of organic systems, in lot of situations the birds began to peck their feathers which finished with a inadmissibility large proportion of cannibalism. As a consequence of this phenomenon, mortality can reach 20% per year (Kristensen, 1998; Kovács & Konrád, 2010). In addition to mortality through cannibalism, mortality caused by predators and the inappropriate behavior of birds by gathering together can sometimes produce suffocation. Thus, the high mortality rate is a real problem, especially from the perspective of welfare and on the consumers eyes (Kovács & Konrád, 2010).

In meat cattle the situation is more complex and organic growth has disadvantages. To ensure a welfare and a natural environment and I mean grazing, that means available place for grazing, as is not available for all farms. And the farms that have space for grazing, also depends on the number of animals, not all of them have space for grazing enough.

If the animals do not graze at all it means that the welfare of the animals is not satisfactory, because it is known that grazing has beneficial effects on the health of ongloans, less severe disorders and better healing and reduces stereotypes and aggression in a herd. Hard concrete and grate, especially when slippery, dirty and wet create serious risks of injury (Anonymous, 2001; Venglovsky, 2010).

In organic agriculture it is not feasible to produce intensive beef due to the recommendation that the daily ration administered should contain at least 60% volume feed and that there should be at least 150 days of grazing per year. Bulls-calf over 1 year old can be accommodated in shelters with access to an outdoor movement paddock all year round. Organic fattening of bulls-calf with concentrates

at discretion is not possible, and it can be questioned whether it is possible to obtain a satisfactory quality carcass with a ration containing 60% volume feed. Fattening of cattle of meat breeds can result in a higher concentration of unsaturated fatty acids in intramuscular fat of meat (Kovács & Konrád, 2010).

Another aspect that have a major impact at meat or milk cattle it's detunning and opinions are for and against in terms of welfare but we must take into account the damages brought in case of non-decornation.

Arguments that are made against the use of ecorn can be viewed through the prism of three major considerations (Vaarst et al., 2004; Pentelescu, 2010):

- the social function of the horns (preserving the integrity of the animals);
- the procedure itself (causes pain and suffering);
- the importance of horns in some breeding systems (biodynamic agriculture).

The major impact of detunning is that the animals can be kept in large numbers in intensive system and lack of self defending with horns. Then, udder hits can have the effect of blood appearing in the milk, resulting in economic and financial implications, because milk can not be marketed and in addition the animal needs medical care. Strong stings in the body can even result in penetrating the abdominal wall or causing abortion (Pentelescu, 2010).

At dairy cows for a farmer the most important aspect is lactation.

During a normal lactation, the cow's organism is confronted with a number of stressors, such as: calving and separation from the calf, involution of the genital apparatus, mechanical milking, the onset of estrus and the installation of a new gestation, lack of active movement, aggressive social interactions, relotizations, at the onset of lactation the energy balance is negative, the phase of mobilization of body reserves, the adaptation of the digestive tract to the increase of feed intake, etc (Gavojdian, 2010).

The second very important aspect for the farmer is to prevent the appearance of lameness. Lameness is the most important condition that

affects the welfare of dairy cows (Chaplin et al., 2000; Szücs & Sossidou, 2010a).

In a study conducted by (Bugueiro et al., 2020) to assess the interconnection between farm welfare, production of milk and reproductive performance in dairy herds on northwestern

Spain. They used Welfare Quality® protocol to collect the welfare grade out of 31 herds. Results shows the production of milk was connected with nonappearance of prolonged hunger norm. Also, good feeding was connected with raise the production of milk. Other factors that were mentioned was nonappearance of pain and injuries. Expression of social behaviors was confirmed too. As we can see, nonappearance of prolonged hunger, pain, injuries, and good feeding are a must.

The next study conducted by Mitrănescu et al. (2020) was made at a farm with dairy cows in the southern region of Romania and have researched the following: health grade of the acropodium via the walk score (Gait Score), condition of animals (BCS - Body Condition Score), hygiene of the body (body hygiene index) and determination in the laboratory of blood serum from 12 Holstein breed cows on different gestation periods of 13 biochemical parameters (BUN, PHOS, URIC, Ca, TP, ALB, GLOB, AST, ALT, TRIG, GGT, GLU and LDH). At the end of the study they concluded: data from the research on the assessment of cows welfare on the farm (GS, BCS and body hygiene) including biochemical blood parameters and physical-chemical and microbiological inspection of milk, was found that the welfare of dairy cows, it's also "average". This study from my point of view, is very complex, even if was made only on 12 Holstein cows but if we are vigilant, we'll see the word "average" at the end. The purpose of the concept animal welfare is for the farmer to fully understand this concept and from perspective of farm welfare not to be at the average level but above, that means greater and exceptional.

But we can ask ourselves: What does it mean animal welfare from farmers point of view? Animal welfare can it be associated with the comfort of the animal? The answer to these questions, we will find it in Figure 3.

Now that we have formed an opinion about animale welfare and more precisely about Welfare Quality® protocol, it should be noted that there is another method to assessment the welfare of animals and it is called ANI 35L.

The "Animal Needs Index" (ANI), was made to apply on the farms like a tool to evaluate and classifying livestock habitation. The process was held in Austria (ANI 35L) and took into account five conditions: (1) possibility of movement, (2) social intercourse, (3) condition of the floor, (4) stabilized climate (counting light and noise) and (5) farmer care. It's applied in Austria, on organic farms and to respect legislation for animal welfare (Bartuseek, 1999). Being careful, we notice that this method of evaluation ANI 35L is used especially in "organic farming" and animal welfare legislation, which warns us that "organic farming" it is not to be neglected.

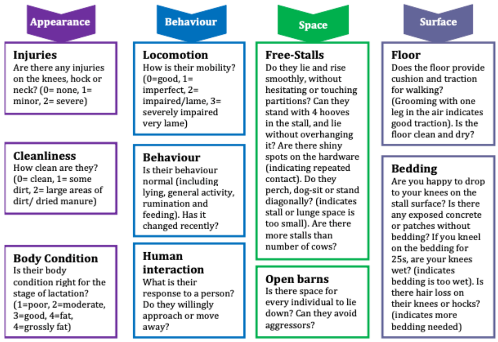

Figure 3. Welfare evaluation results for comfort of dairy cow (DCWS 2012; compassioninfoodbusiness.com "Welfare of dairy cow", 2013)

In a study that was made on dairy cows farm in south-eastern Romania to assessment of the welfare level (Funaris et al., 2016), to establish the welfare level, they used ANI 35L/2000 - Austrian Animal Needs Index. The details from the study was this: 26 objective was scored to observe 5 areas of influence for welfare indices: mobility, social interactions, floor, air and light, stockmanship. Then they came to this conclusion: Indicators with high score was those in the areas with social interactions and mobility. Areas with low score was air and light, floor and also, outside areas cleanliness. Even if, negative indicators was more than positive indicators, the score of ANI 35L was (30.5 points) and shows a great welfare. To raise production of milk, all the negative indicators must be optimize, especially light in the shelter and cleanliness on the areas outside.

We are already seeing differences between Welfare Quality® protocol and ANI 35L. About Welfare Quality® protocol it can be mentioned that it is a subjective way of

appreciation and ANI 35L it is an objective way of appreciating.
Another aspect for dairy cows welfare is pasture access or grazing.
In a review about continuously housed and pasture based production system of welfare for dairy cows, Arnott et al. (2017) examined three major topics: health, behavior and physiology. And they concluded: The cows maintained on pasture based system had better health compared to the cows maintained in continuously housed system. In terms of behavior, pasture access had benefits on grazing, lying, resting and low grade of aggression. Negative aspect was mentioned to pasture based on physiology to affect the welfare on inclement weather. The outcome from the review emphasizes the benefit of including pasture access for dairy production and welfare.
According the review of Arnott et al. (2017) the most important aspects regarding dairy cows welfare within each topic was:

1) Health
• Lameness;
• Hock lesions;
• Mastitis;
• Uterine disease;
• Other infectious disease;
• Endoparasites;
• Mortality.

2) Behavior
• Feeding/grazing;
• Lying/resting;
• Aggression;
• Behavioural knowledge gaps;
• Cow preference.

3) Physiology
• Thermal stressors;
• The impact of sunlight.

Another study made on Denmark that used Welfare Quality® protocol, to see the impact of grazing on the welfare for dairy cow herds (Burow et al., 2013), concluded that many daily grazing hours was more beneficial compared to few daily grazing hours considering welfare of dairy cow herds.
Although, when we talk about farm animals, we refer to products and by-products (meat, offals, eggs, milk and milk derivatives), we must not forget to mention the horses and their importance.
The horse evolved as an animal raised in a group on pasture in freedom and domestication resulted in some conflicts with this evolutionary path.
Horses occupy a special position among farm animals. Currently, huge percentage of the world's population of horses are still used for labor in developing countries, but this mode of use is generally decreasing and horse has gained the principal role in the developed part of the world for pleasure either sport. On world there is considerable diversity in terms of use, accommodation, management and horse breeds, but one can hardly talk about the industrial breeding of the horse. Even in large farms there is a tendency to raise a small number of animals in one shelter for several reasons. Horses are very valuable animals and many of them travel nationally and internationally to and from competitions, studs and auctions. This exchange provides ideal possibilities for disseminating the agents of infectious diseases from respiratory problems to abortion. Also, horses are kept for a long time with other farm animals and it is more advantageous to be separated by groups of use and age. Moreover, the horses are very sensitive to the air quality in the shelter and failure to ensure this requirement can seriously affect the performance of the horses (Perry, 2006; Venglovsky, 2010).
The hygiene and physical environment of a shelter can affect the welfare of horses through (Venglovsky, 2010):

• Increasing the magnitude of aggressiveness of microorganisms, parasites, harmful gases and allergenic and irritating particles;
• Alteration of the local or systemic resistance of the horse;
• Increasing the risk of physical injury;
• Inability to ensure the behavioral requirements of the horse.

The welfare problems of horses raise the greatest public concerns about this species. Welfare problems exist during the growth and fattening of these pathways, such as poor quality of accommodation, lack of free movement, improper handling, insufficient veterinary care, weaving and care of the hooves at irregular intervals of time, and others (Venglovsky, 2010). A special chapter of welfare problems is that during transport to slaughterhouses and waiting until slaughter. All over the world thousands of horses are transported to slaughterhouses, sometimes very long distances. Preferably are young animals up to the age of 18 months. The usual problems that arise are overcrowding in vehicles, prolonged transport, handling, watering and inadequate veterinary inspection.

On EU, animals welfare during transport is stipulated in the Council Regulation 1/2005.

The last aspect to be noted is the interaction of man with animals.

Several studies have shown the influence of the behavior of caregivers on the behavior of animals. Animals that are gently treated by caregivers are less fearful in contact with humans and are easier to handle during weighing, when driven with the help of the capastre or transported (Lensink, 2001)

Understanding the perception that dairy cows have about human contact, such as palpation of different body regions, is important for improving the human animal relationship, for the welfare of animals, as well as for milk production (Schmied et al., 2008).

Improving the human animal relationship is important because it has beneficial effects on animal welfare and productivity, along with working conditions and the safety of caregivers (Rushen et al., 1999; Hemsworth, 2003).

In the man animal relationship, fear must necessarily be avoided.

Fear has significant effects on productivity and welfare in farm animals, mainly when animals are afraid by the presence of caregivers (Hemsworth & Coleman, 1998).

Depending on the events in the environment, correlated with the state of the organism at a certain time, the animal manifests a certain mood. If everything unfolds normally the animal is quiet. When pleasant events occur (feeding, watering, caressing) the animal shows good mood. The malaise presents different forms of exteriorization such as greed, the tendency to defend or flee, etc. and is due to hunger, fear or pain (Stanciu, 1999).

As we can see, the interaction of human animals is of major importance. Therefore, caregivers must be trained and made aware of the importance of their relationship with animals.

CONCLUSIONS

According to Welfare Quality® protocol and ANI 35L, animal welfare is gaining ground on farms and many farmers are adapting to these systems. A very important factor remains the human-animal interaction, and the caregivers must be trained not to induce fear in animals. Finally, after Welfare Quality® and ANI 35L are applied on farms, it remains the task to inform the consumer in a pleasant way and to accept his own decision, to pay extra for products coming from farms where animal welfare is a priority.

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THE ROLE OF CIRCULARITY IN MIXED FARM SYSTEMS

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Abstract

The paper aimed to present the practice of mixed farming systems which is based on circularity and proposes stronger links between crops and livestock. It aims to emulate the nutrient circuits resulted from food production based on those in natural systems, shifting from linearity towards cyclicity to reduce anthropic impact. The paper analysed the selected articles in order to identify strategies implemented in mixed farming systems which improve circularity and have positive effects on the environment and presented the results and discussions section in the form of guidelines and based our logic structure on the principles of circularity in agricultural systems. It is recommendable that farmers and stakeholders try to preserve and, where possible, increase the biotic diversity of agricultural landscapes, both in terms of crops and livestock (for example, by using cover crops and intercropping), but also in terms of habitats that can provide beneficial ecosystem services (such as: shelter for natural enemies, nitrogen fixing plants, etc.).

Key words*: circularity, livestock, recycle, waste management.*

INTRODUCTION

Predictions on the dynamics of worldwide human populations indicate a strong increase in the years to come (McKee et al., 2004). Additional food resources will be needed to support growing human populations, which will likely increase the anthropic impact generated by food production activities. The development of better conceptual and practical approaches is much needed for more sustainable food production.

Crops and livestock represent the principal means of food production and, also, some of the major contributors to ecosystem changes. Secondary products of these activities, such as greenhouse gases and other pollutants are known to have daunting effects on ecosystems, especially due to their long lasting and large-scale effects. Better management practices in agriculture are needed to reduce the impact of climate changes, without compromising socio-economic stability (De Boer & Van Ittersum, 2018).

In search of solutions, inspiration can be drawn from self-sustainable natural ecosystems that maintain their balance based on complexity and circularity. Mixed farming systems have been proposed as one of the viable solutions aimed towards sustainable food production (Oomen et al., 1998). The practice of mixed farming systems is based on circularity and proposes stronger links between crops and livestock. It aims to emulate the nutrient circuits resulted from food production based on those in natural systems, shifting from linearity towards cyclicity to reduce anthropic impact.

Mixed farming systems have been historically associated with subsistence farming that currently characterize underdeveloped or developing countries (Thornton & Herrero, 2014). The increased awareness of sustainability requirements and the possibility of reduced impact prompted more awareness and research on how this farming practice can be improved and implemented in the current context of food production, along with other environmentally friendly measures.

We adhere to this research direction and aim to better understand the issue of greenhouse gas emissions in mixed farm systems, in order to help structure the scientific knowledge on this topic and, ultimately, to contribute to the overall development of and ease of use for beneficial farming practices.

MATERIALS AND METHODS

We searched for articles related to the topic of greenhouse gases in mixed farming systems on

the Web of Science and Google Scholar platforms. We analysed the selected articles in order to identify strategies implemented in mixed farming systems which improve circularity and have positive effects on the environment.
We presented the results and discussions section in the form of guidelines and based our logic structure on the principles of circularity in agricultural systems (Oosting et al., 2021) and mainly focus on the research carried out in temperate regions.

RESULTS AND DISCUSSIONS

The concept of circularity in agricultural systems is based on multiple cornerstones. These represent concepts needed to be applied in order to reach a more sustainable agriculture and can be described as follows: (1) use arable land to produce food directly for human populations; (2) reduce food waste as much as possible; (3) recycle the by-products of agriculture and the use of animals with low opportunity cost as new sources of food, manure and for ecosystem services (de Boer & van Ittersum., 2018; Oosting et al., 2021). The implementation of these principles, along with other practices and methods, seem to have the potential to reduce greenhouse gas emissions resulted from food production activities. By definition, mixed farming systems already account, at least partially, to the aforementioned principles. We will further address how these principles could be further included in this farming practice, in order to benefit from a more sustainable way of food production.
The principle of using the land to predominantly grow food directly for human population (1st principle) could be applied in mixed farming systems at landscape scale, by partitioning the crop selection. Further efficiency might be achieved by an adequate rotation of the human consumption designated crops with high importance for the quantity and quality of the yield (Bowels et al., 2020; Shah et al., 2021) and by the practice of intercropping, which is also documented to increase the yield up by 20% (Yu et al., 2015). Large scale diversified crop rotation and intercropping were also observed to enhance crop resilience and soil biodiversity (Bowels et al., 2020; Yu et al., 2015), thus representing viable practices to be used in the context of climatic changes. Furthermore, mixing cereal crops along with legumes, especially N fixing taxa, has been documented to be efficient in fixing N in the soil and for reducing its emission into the atmosphere (Rauw et al., 2022). The principle of using agriculture to produce food primarily for human consumption is also inextricably linked to the feeding preference exhibited within societies (De Boer and Van Ittersum, 2018). For increasing the circularity in agriculture, it is recommended that consumers should adhere to a more plant-based diet, in order to reduce the greenhouse gas emissions resulted from animal breeding and consumption (Yue et al., 2017).
Reducing waste (2nd principle) is another principle which is important for reducing greenhouse gas emissions associated with food production and represents a basic, yet hard to reach desiderate. This principle can be applied for all the parties implicated in the cycle of food production, ranging from the producers to the intermediaries (processing units, markets) and the consumers. More research is still needed to increase the efficiency of nutrient enrichment, pest control and livestock waste management in agriculture and in mixed farming systems. With respect to the crops, so far, the best practices include the use of plants and cover crops in order to enrich the soil with nutrients which are beneficial for the quality and the productivity of crops (Snapp et al., 2005), and thus reduce nitrogen and phosphorous waste and further pollution. The use of green peas cultures, for example, along other crops can be used in order to fix N into soil and enrich it with certain nutrients. Research further suggests that cover crops can be beneficial for the management of agriculture derived N emissions, by reducing the leeching of NO_3 in multiple instances (Gabriel et al., 2012). The additional vegetal waste resulted from cover crops could be further used in accordance with the third enounced principle and be incorporated into the diet of the livestock. As such, the choice of suitable plants is very important in the waste reduction strategy. Pest control improvement represents one challenging aspect in the context of circular

agriculture, especially when discussing mixed farming systems. The traditional use of pesticides is associated, among other, with increased health problems in human populations, environmental pollution (the persistence of synthetic compounds into the ecosystem fluxes) and with the reduction of local and landscape biodiversity (including the decline of the useful group of natural enemies). Though advantageous in terms of pollution reduction, the use of biotic pesticides still poses inherent problems, with the most notably being the biodiversity reduction potential. On the other hand, the use of natural enemies is a practice with no significant deterrent side (Sunderland, 1999). Furthermore, this practice is closely linked to the concepts of circularity in the processes of food production, since there are no existing by-products. The efficient use of natural predators requires a landscape level approach and certain concessions in terms of land use, crop selection, cropping strategy and tillage practices. The diversity of natural enemies (both in terms of community structure and functionality) is important in order to efficiently prey on and control the variety of crop pests which may inhabit complex agricultural landscapes. Research documents that the diversity of habitats within agricultural landscapes serves to increase the diversity of natural enemies. This is achieved by increasing the natural vegetation strands, both forested and herbaceous (Zamberletti et al., 2021) and by increasing the diversity of different crops (Redlich et al., 2018). In addition to landscape heterogeneity, other agricultural practices, such as intercropping and cover crops were also documented to increase the overall predatory of invertebrate pests in agricultural landscapes (Boweres et al., 2021). The limitations in pest control exhibited by the communities of natural enemies could be supplemented by the use of pesticides. However, this aspect requires further research and developments, as it is widely documented that in multiple instances, the pesticide use has proven to be harmful to the natural enemies, in addition to the pests it is applied for (Beers et al. 2016).

The last principle of circularity and sustainability (3rd) in agriculture proposes the recycling of agricultural by-products and the use of animals with low opportunity cost, as new sources of food, manure and for ecosystem services. To account for the first half of the principle, the resulted crop waste (vegetal biomass) could be reincorporated into the food production cycles by various means, such as readily available compost or animal food. Soil nutrient enrichment can be achieved, at least partially, by applying compost resulted from the vegetal waste of previous crops, with possible even better applications in regions with lower soil quality (De Boer & Van Ittersum, 2018). Research suggests that in order to increase circularity of food production and consumption, it is beneficial to incorporate a medium amount of vegetal waste fed animals in our diet (Van Zanten et al. 2018). As such, the second part of the principle suggests that the increased use of low-opportunity-cost feed animals is linked to reduced supplemental consumption and residues linked to more expensive food. Multiple vegetal waste can be processed and incorporated in the diet of livestock. We consider that mixed farming systems provide an ideal platform for such practices, as they are very convenient for the construction of processing units that could transform vegetal and animal waste into food and fertilizers. Again, large scale project conception and management represents a perquisite for an efficient implementation of this principle and ultimately attaining more circularity in mixed farm systems. Additionally, more research and legislative developments are required to better use farm waste, especially for that of animal origin. The current EU legislation almost entirely prohibits the use of animal waste as a food source, with few exceptions for blood, in order to limit potential diseases (Zu Ermgassen et al., 2016). In order to avoid disease spreading, more research and investments are needed into processing and sanitation technologies, ranging from machine engineering to microbiology and chemistry. Strict control protocols and analysis should also contribute to safe use of waste and by-products. The capacity of farmers and control agencies to limit potential disease spreading is closely linked to the increase of circularity in mixed farming systems.

CONCLUSIONS

Large scale multidisciplinary approach is essential in order to attain a higher level of circularity in mixed and other type of farming systems. Though theoretically ideal, in practice full circularity, as encountered in natural ecological systems, cannot be attained in agricultural ecosystems and landscapes. Mixed farming systems represent a valuable platform for the research, innovation and improvement of circularity in food production. Using the residue and natural by-products resulted from crops and livestock in support of each other, can help reduce waste, especially in terms of pollutants resulted from fertilizers and pesticides. It is recommendable that farmers and stakeholders try to preserve and, where possible, increase the biotic diversity of agricultural landscapes, both in terms of crops and livestock (for example, by using cover crops and intercropping), but also in terms of habitats that can provide beneficial ecosystem services (such as: shelter for natural enemies, nitrogen fixing plants, etc.). Lastly, an increase in the research and technology, coupled with shifting preferences and expectations in food production and consumption can enable us to move towards more circular food production and sustainability in agricultural landscape management.

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ANALYSIS OF SOME BEHAVIORAL REACTIONS OF KARAKACHAN HORSES

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Abstract

The observation was made on a herd of Karakachan horses, bred freely in the area of the village of Prisadets, Southern Bulgaria. The study covers a period of one year, with 24-hour field surveys conducted every month. The analysis shows that horses drink water relatively rarely. Although not statistically proven, the highest percentage of horses drink water at moderate ambient temperatures - about 22°C, while at high temperatures around 36°C, when standing in the shade, the percentage of horses drinking water drops to 46.15%. Karakachan horses spend most of their time grazing. Most horses graze at noon (91.28%), and the least at night (40.90%). The factor time of the day had a significant effect on the following traits of behavior: grazing (P <0.001), sleeping / resting lying down (P <0.001), sleeping/resting standing (P <0.01), standing on alert (P <0.01) and standing in the shade (P <0.01). Ambient temperature affects resting behavior: lying down (P <0.01) and standing (P <0.01), chasing insects with a tail and head (P <0.001), and standing on alert (P < 0.001).

***Key words**: autochthonous breed, behavior, Karakachan horse.*

INTRODUCTION

The Karakachan horses are an old Bulgarian autochthonous breed, preserved in relatively the same form in which it existed over the centuries (Petrov, 1940; Sabeva, 2009; Popova & Etarska, 2020). In addition to use for typical agricultural and commercial purposes, with careful management, the Karakachan breed is suitable for use and maintaining habitats and supporting rare and protected bird species, such as the Imperial Eagle and Lesser Kestrel (Krastev et al., 2020). In this regard research on the behaviour of the karakachan horses will enable us to collect data on a breed that has preserved genes from ancient times.

Horse breeding is a specific branch, and the ethological knowledge in the field is mainly empirical in nature. This knowledge is of great importance, but its drawback is that it is not scientifically substantiated for a number of phenomena, as well as for a number of regularities, which should serve as a basis for creating an optimal growing environment (Petkov et al., 1999; Hoffmann et al., 2012; Yarnell et al., 2015; Löckener et al., 2016; Rochais et al., 2016; Sigurjónsdóttir & Haraldsson, 2019). For this reason, the intervention of a person who does not know the behavioral manifestations of horses can lead to serious failures. Conversely, if man is familiar with their ethological features, he can discover new ways to increase their productivity and discover new benefits from their use (Hausberger & Muller, 2002; Hausberger et al., 2008; Thompson et al., 2015; Hall et al., 2018; Butler et al., 2019; Kelly et al., 2021).

The purpose of the research is to study basic ethological, functional signs of karakachan horses from the national gene pool in ecological and biocompatible breeding.

MATERIALS AND METHODS

The study was conducted in the area of the village of Prisadets (on the Bulgarian/Turkish border - within Bulgaria) falling within SPA Sakar (BG0002021), which overlaps with SCI (BG0000212), part of the ecological network NATURA 2000 (MOEW, 2013). In biogeographical terms, the area falls into the Southern biogeographical region and, more specifically, according to the biotic basis, it refers to the "Dolnomarishko - Dolnotundzhansky" sub-region (Gruev &

Kuzmanov, 1999), as Mediterranean influence penetrates the sub-region along the Maritsa and Tundzha rivers. This defines the climate as milder and allows the horses to be kept outdoors all year round.

Object of observation was a herd of horses of the Karakachan breed, which are free-range horses all year round - 29 mares, 1 stallion and 10 foals. The observations were carried out periodically within 12 months. Spring, summer, autumn and winter 12 and 24-hour field observations were conducted. Both the periods with the most typical climatic and forage conditions for the given season, as well as the days with extreme temperatures, humidity, etc., were chosen.

The behavioral indicators we monitored were selected during the first few months of the project. A farm was chosen for breeding free-roaming animals of the Karakachan horse breed. Based on the research, we identified the following signs and factors influencing them, which will be included in the study:

- ✓ Grazing;
- ✓ Drinking water;
- ✓ Rest - sleeping/resting while standing up, sleeping/resting lying down;
- ✓ Mares in heat - frequent urination, covering of mares by stallion;
- ✓ Insect protection - rolling, self-cleaning, tail wagging/head tossing to remove insects;
- ✓ Seeking shelter and comfort - standing with back to trees/bushes that stop the wind, back to the rain and head down, cuddling (horses are under mothers), sun bathing, standing in the shade;
- ✓ Standing alert.

Seven thousand seventy-six (7076) recordings of various behavioral responses were processed for all observations.

The data from the field observations of the horses' behavior were processed variationally-statistically, by using multivariate analysis of variance according to models with the following structures:

Yijk=μ+Zi+Ko+Wv+SXvi+MNoi+eiov(vi)(oi) (M1);
Yijk = μ+Mi+Tj+ZFji+eij(ji) (M2),

where: Yijklm - observation vector; μ - general average constant; Zi is a fixed effect of the season (i = 4); Ko - climate (o=5); Wv - time of day (v = 5); SXij - time of day in the season (ji = 5); MNoi - climate in the season (oi = 5); Mi - month (n = 11); Tj - enviromental temperature (n = 43); ZFji - temperature in the month (n = 42); eijk .. - residual variant.

Statistical processing was performed with the SPSS 21 program.

RESULTS AND DISCUSSIONS

Native breeds of horses are free-range in herds, and increasing the number of horses in the herd leads to changes in both individual and group behavior.

Based on the records collected and processed from the observations, the influence of some paratypical factors on the behavior of the horses was calculated (Table 1 and Table 2).

Table 1. Influence of some paratypic factors on the animal behavior - Model 1

Animal Behavior Activities	Factors, F-criteria and degree of statistical sig-nificance									
	Model 1									
	df	season	df	climate	df	time of the day	df	time of the day in the season	df	climate in the season
Grazing	3	3.433*	4	10.931***	4	6.785***	10	8.133***	5	1.376
sleeping /resting lying down	3	2.906*	4	6.377***	4	8.753***	5	0.202	2	2.715*
Sleeping /resting standing up	3	23.144***	4	13.838***	4	4.734**	9	3.138**	5	7.841***
chasing insects with tail and head	3	4.538**	4	2.745*	3	8.985***	6	3.970**	4	4.787***
standing on alert	3	1.213	2	0.876	3	5.305**	1	0.745	3	1.524
standing in the shade	1	0.796	2	0.646	3	3.480*	1	0.659	1	1.962
drinking water	3	22.665***	2	25.424***	3	0.778	3	0.197	1	0.362
salt licking	2	19.653***	2	1.992	2	9.346**	2	0.185	1	0.245
hiding in the woods	2	7.524***	3	3.111*	3	9.511***	1	4.565*	1	0.752
sunbathing	1	17.821***	1	7.692*	1	10.256*	1	1.321	1	1.023
mutual cleansing	3	16.188***	4	1.119	3	0.259	1	0.383	2	1.913
mares in heat	2	5.009*	4	0.616	2	2.214	2	0.978	1	1.232

***P< 0.001; **P<0.01; *P<0.05

From the obtained results, it can be seen that all factors have a reliable influence on the trait of chasing insects with a tail and a head. Only the factor climate in the season has a reliable influence on the grazing sign. The factors month, season and climate have a reliable influence on the drinking water (P<0.001). The time of day in the season significantly affected only the traits grazing ($P<0.001$), sleeping/resting while standing up ($P<0.01$), tail wagging and head tossing to remove insects ($P<0.01$), and hiding in the forest ($P<0.05$). The factors time of day ($P<0.01$), temperature ($P<0.001$) and temperature in the month ($P<0.01$) have a reliable influence on alertness. The sign drinking water is reliably influenced by the factors season ($P<0.001$), climate ($P<0.001$) and temperature ($P<0.001$). The factors season, month and temperature have a reliable influence on the signs of grazing and mutual cleaning, and on the sign of salt licking - the season ($P<0.001$) and time of day ($P<0.01$).

Table 2. Influence of some paratypic factors on the animal behavior - Model 2

Animal Behavior Activities	Factors, F-criteria and degree of statistical sig-nificance					
	Model 2					
	df	месец	df	температура	df	температура в месеца
Grazing	8	13.116***	42	3.771***	41	2.773***
sleeping /resting lying down	9	1.412	29	3.254***	9	5.009***
Sleeping /resting standing up	9	4.715***	37	2.568***	19	0.996
chasing insects with tail and head	9	2.597**	37	2.679***	21	1.321
standing on alert	7	9.770***	33	7.684***	19	4.117***
standing in the shade	6	1.396	22	3.382***	2	4.932**
drinking water	1	1.817	16	3.620***	1	0
salt licking	6	11.543***	15	1.516	3	0.385
hiding in the woods	3	8.123***	29	1.353	8	0
sunbathi ng	4	3.676*	14	3.037**	1	0.655
mutual cleansin g	7	5.311***	13	9.498***	3	4.154*
mares in heat	3	7.789**	11	3.935**	2	2.625

***P< 0.001; **P<0.01; *P<0.05

Figure 1 shows the dynamics of the different behavioral responses during the different periods of the day. Of the observed horses, 31.25-38.44% graze in the morning until the afternoon, with this percentage decreasing to 25.98% in the evening and 19.52% at night. From these results we can draw the conclusion that horses graze around the clock. Horses stand in the shade most during the hot hours of the day, namely in the afternoon - 49.89%, while in the morning and at noon this percentage varies from 21.53% to 27.34%. The highest percentage of horses standing alert is during the evening hours - 40.59%, when it starts to get dark and the jackals start howling. After midnight, this percentage decreases to 38.65% and by morning it reaches 7.50%.

Thanks to the special construction of the tendon apparatus of their limbs, horses can sleep while standing up (Petkov et al., 1999). In the herd observed by us, the horses sleep/rest while standing up throughout the day, with the lowest percentage in the noon and afternoon (16.64-17.05%) and morning (23.37%) hours, and in the evening and night it rises slightly to 30.66% respectively and 39.40%. The percentage of horses sleeping/resting while lying down is highest in the afternoon - 8.11%, in the evening and night it decreases to 1.9%, and in the morning and noon hours it increases to 6.36% and 6.76%, respectively, especially on warm days. From the obtained results, it can be seen that a greater percentage of the observed horses are awake during the day, and at night they rest more, taking turns in groups that stand alert.

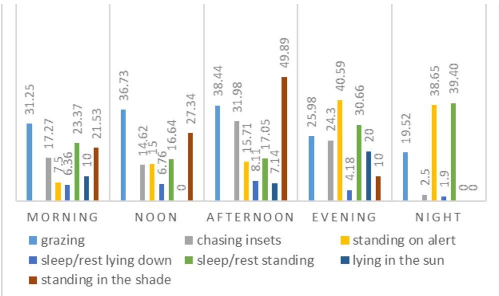

Figure 1. Dynamics of different behavioral reactions during different periods of the day, %

Figure 2 shows the dynamics of the different behavioral responses during the different seasons. From our field observations, it can be seen that in the spring season, when the weather starts to warm up and the end of winter has come, as well as the lack of grazing, the highest percentage of horses perform the activities of grazing (37.95%), drinking water (45.56%), sleeping/resting lying down (10.37%) and licking salt (51.67%). In addition, in the spring season, the highest number of mares in heat are observed - 8.36%.

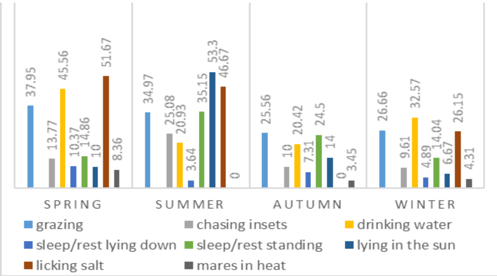

Figure 2. Dynamics of different behavioral responses in different seasons, %

In the remaining seasons, the percentage distribution of these behavioral reactions gradually decreases, with the lowest percentage in the fall of grazing - 25.56%, salt licking - 0%, water drinking 20.42% and mares in heat - 3.45%. During the summer months, due to high temperatures and warm weather, the percentage of sun-bathing horses (53.3%), sleeping/resting while standing up (35.15%) and tossing their heads and wagging their tails to chase away insects increases - 25.08%.

In the spring and summer season, horses hide in the forest more often than in autumn and winter. From our observations, we found that on extremely hot days when there is no wind, the horses prefer to hide in the forest. The most likely cause is not the heat, but the presence of insects. The wind brings a cool feeling and reduces insects, which makes the horses more comfortable and able to graze more of the day. It can be concluded that horses are relatively more resistant to high temperatures, strong wind and sunny days than to the presence of insects.

In their study, Górecka and Jezierski (2007) tracked the defensive behavior of Konik horses in response to insect harassment. They found that horses stood closer together (bunched, head-to-tail position) in hot weather and windless days when insect harassment was apparently stronger. In our study, we found no such correlation in Karakachan horses.

Figure 3 shows the effect of temperature on the dynamics of insect chasing and water drinking. Although it has not been statistically proven (Table 1) the highest percentage of horses drink water at moderate ambient temperatures - around 22^{0}C, while at high temperatures around 36^{0}C, when standing in the shade, the percentage of horses drinking water drops to 46.15%. Due to the high temperatures and presence of more insects in summer, 93.96% of horses wag their tail or toss their head to repel insects when temperatures rise to around 37^{0}C.

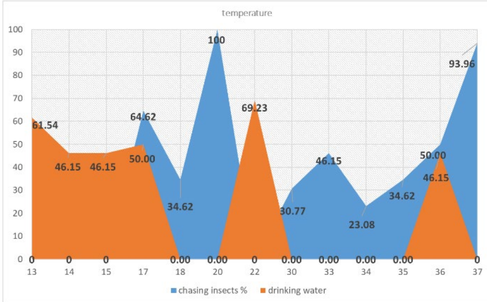

Figure 3. Effect of temperature on the dynamics of chasing insects and drinking water, %

Analogously to the greater presence of insects in the summer months, the largest percentage of horses protect themselves from insects in August, while in the autumn months this behavior decreases with a decrease in environmental temperatures and a decrease in insects, reaching 39.13% in the month of November.

The observed influence of weather conditions on insect pestering activity was similar to those observed by Strickman et al (1995), Górecka and Jezierski (2007), i.e. more frequent defensive behaviors such as tail wagging, head tossing, etc. with the Karakachan horses, we observed during hot, sunny and windless days.

According to Parvanov et al. (1997) horses are seasonal-polycyclic animals, where the breeding season in our latitudes lasts from February to June, and sometimes in August-September. In some mares, the sexual cycle repeats throughout the year, and in others for a short period of the year, depending on the climate, geographical location, environmental conditions, work load, etc. Sex functions in the spring bear the imprint of the conditions of rearing and feeding in the winter, the length of daylight and temperature. Ginter (1974) indicated that mares raised in northern latitudes were in heat from early May to October.

As can be seen from the present study (Figure 4), in freely bred horses we observe mares in heat in the temperature range from -5^{0}C to 33^{0}C, with the highest percentage of mares in heat between 15^{0}C and 21^{0}C. The earliest we observed mares in heat was at the end of

February, and the latest was until the middle of November. During the too cold (December, January) and too hot months (June, July, August) no coverings and mares in heat were observed.

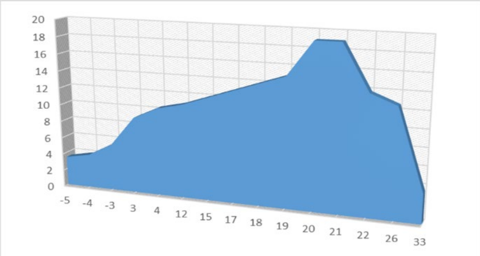

Figure 4. Influence of temperature on the mares in heat, %

From the field observations, we found that on very windy days and during precipitation (rain, snow) the horses do not enter the forest, but find bushes or individual trees to stand next to and turn their croup towards the wind and/or rain /snow (Figure 5). If the snowfall is light, it does not prevent the horses from sleeping/resting lying down.

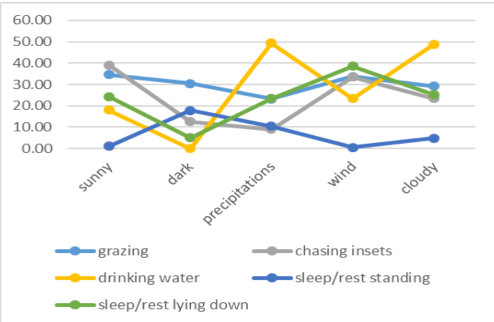

Figure 5. Influence of the climate on some behavioral reactions of horses, %

In the social behavior of herds, the individual distance between individual animals is also important. The term "individual distance" refers to the normal distance between two individuals of a given animal species. Observance of individual distance in the group of animals living together is one of the basic conditions for harmonious existence. The primary biological reason for this is assumed to be the provision of sufficient food space. (Petkov et al., 1999).

In unfavorable climatic conditions, we observed a shortening of the distance between the horses themselves (they stand closer to each other or huddle together), the purpose of which is to keep a bond and keep warm. Despite the weather conditions, we never once saw the horses using the sheds available. From this, it can be judged that the horses (especially the Karakachan horses), which are bred freely all year round, do not feel comfortable confined in buildings or under sheds. When there is no wind or rain, at too short a distance, the horses start biting and kicking each other. In good weather conditions, the horses in the herd maintain a greater distance from each other and spread out over the pasture. During the spring and summer observations, and less often during the winter, the horses approached unfamiliar objects (a car) and began to lick it. The most likely reason is their need for salt and they lick the stuck salt on the unknown object, which in this case is a car (Figure 6). At the watering place there are many large blocks of salt scattered around the field, but apparently the horses don't go there that often or spend enough time there. Because this herd is raised on a very large area and there is no way to throw salt blocks around, the horses get it by licking objects (the car) or by licking each other, especially in the warmer months.

Because horses are social animals, even free-range horses are curious and use sniffing, groping, and approaching unfamiliar people/objects as a way to learn about their surroundings.

Figure 6. Horses licking a foreign object for the purpose of familiarization and obtaining salt

In every single field observation, despite our efforts to keep a distance so as not to disturb their usual activities, after 2-3 hours of observations, the horses came to us. With each subsequent visit, the approach time shortened.

The horses got used to the human presence relatively quickly and there was no presence of aggressive behavior on their part.
The results of the present study correspond with those indicated by Jastrz̨ebska et al. (2021), who studied the behavior of stabled and free-range Konik geldings and mares during standard behavioral tests considered as determinants of willingness to explore. According to the authors, free-range horses were not only less likely to stray from the herd, but were also more likely to let their muzzles be stroked than stabled horses. In addition, they showed their curiosity to learn by approaching new objects overcoming their fear. This may further indicate that free range horses demonstrate a greater willingness to explore than stabled horses.

CONCLUSIONS

From the observation we can draw a conclusion that the horses graze around the clock.
A greater percentage of the observed horses are awake during the day and rest more at night, taking turns in alert groups.
It has been found that horses are relatively more resistant to high and low temperatures, strong wind and precipitation than to the presence of insects.
In bad weather conditions, a shortening of the distance between the animals themselves is observed. Despite the weather conditions, we never once saw the horses using the sheds available. From this, it can be judged that the Karakachan horses, which are bred freely all year round, do not feel comfortable enclosed in buildings or under sheds.
Due to the high temperatures and presence of more insects in summer, 93.96% of horses flick their tail or head to repel insects when temperatures rise to around 37^0C.
In free-range horses, we observe mares in heat in the temperature range from -5^0C to 33^0C, with the highest percentage of mares in heat between 15^0C and 21^0C.

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RESEARCH ON THE LACTOGENIC POTENTIAL IN THE RESULTING F_1 SHEEP FROM THE CROSSING OF LOCAL SHEEP FROM NORTH-EASTERN AREA OF ROMANIA WITH AWASSI RAMS

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Abstract

The aim of the present work was to analyze the lactogenic potential in the second lactation of the F1 crossbreeds resulting from crossing the local sheep from the north-eastern area of the country with Awassi breed rams. To determine the total milk production, the control of milk production includes the suckling period of the lambs and the milking period of the ewes. The AT4 method was used in the milking period following the technical specifications recommended by the ICAR. During the suckling period, the amount of milk in crossbreds F1 sheep was 49.72 kg, and the production of milked milk was 95.13 kg. Average daily milk production on the 4 controls for F1 crossbred ewes was 774.37 ± 9.76 g with limits between 542 and 1006 g milk. The total production of milk obtained in 180 days at the second lactation of crossbreds F1 sheep was 144.85 kg, being 3% lower compared to that obtained by purebred Awassi sheep. The results regarding milk production at the second lactation of the F1 crossbred ewes are very good, so we recommend to breeders the use of crossing local sheep from the north-eastern area of the country with the Awassi breed for a significant improvement in milk production.

Key words: *Awassi breed, crossing, latogenic potential, local sheep, milk production.*

INTRODUCTION

In Romania there is a very large number of small family sheep holdings (73% of sheep holdings used an agricultural area of 0-5 ha in 2016 and 63% in 2020), where the number of animals is very small, which it leads to the production of milk and meat intended mainly for own consumption and less for their delivery to the market, hence the subsistence character of these holdings (NIS, 2022).

In these holdings, no improvement program is applied, sheep are raised from non-improved local breeds, which are exploited especially in the extensive exploitation system, based on as economical a maintenance as possible, i.e. by using for a long period of time during a year of grazing, and during the short stable periods (90-120 days) the growth is done in simple shelters, with the vast majority of the work done manually. As a result, the productive and economic performances achieved by these animals are lower compared to those achieved by animals from western European countries.

In these western European countries, emphasis is currently being placed on the breeding of high-performance sheep breeds, which respond more effectively to the ever-changing demands of the European market, for high-quality products obtained from the sheep species (Padeanu & Voia, 2010; Răducuță, 2022).

Taking into account the aforementioned, as well as the interest shown by sheep breeders in the recent period for improving production and implicitly increasing the profitability of these holdings, we proposed to improve milk production by crossing sheep from the north-eastern area of the country with rams from the Awassi breed. This specialized breed of dairy sheep was imported to our country in the 80s and proved to have a good adaptability to the extensive exploitation system practiced in the north-eastern area of Romania (Pascal, 2015; Tafta et al., 1997).

The aim of the work is to increase the milk production of native sheep from the north-eastern area of the country, where the reproductive activity was carried out uncontrolled, without a pre-established improvement program, by absorbing crosses of sheep from local breeds with purebred rams Awassi to increase productivity in sheep farms.

MATERIALS AND METHODS

Absorption crosses of local ewes with Awassi rams began in autumn 2018, and during 2022 the F1 crossbred females were in their second calving (Popescu, 2020). The objective of the research was the evaluation of F1 females in relation to the quantitative and qualitative production of milk in the second lactation.

The working method used was suitable for objectives of this kind of research. To determine the total milk production, the control of milk production included the suckling period of the lambs and the milking period of the ewes.

The amount of milk during the suckling period was estimated by the method of valorization of the suckled milk of the lamb, respectively by the weight gain achieved, using the transformation coefficient method. Considering the particularities of the nutrition of the suckling lambs, the assessment of the amount of milk sucked from the mother sheep was made for 2 periods, namely:

• the suckling period from calving to the age of 28 days - period in which the amount of milk sucked for 1 kg of weight gain is 5.5 liters;

• the suckling period from the age of 28 days of lambs to their weaning – respectively at the age of 60 days in which the amount of milk sucked for 1 kg of increased weight gain is 4.5 liters.

The amount of milk milked. Performance evaluation for milk production during the milking period was based on the application of successive productive controls (n = 4 controls), at intervals of 30 days. At each control interval, the standard method was used, namely AT4 (alternative control) respecting the technical specifications recommended by the International Committee for Animal Recording (ICAR).

The control of milk production during the milking period was carried out by the controllers of A.J.C.O "MIORITA MOLDAVIS" Bacău in two sheep holdings, respectively a private holding in Iași county (F1 females in the second lactation) and within SCDCOC Secuieni Bacău (Awassi females breed in second lactation).

The milking of the ewes taken in the study during the control period (n = 200 heads; batch 1 F1 ewes in the second lactation = 100 heads; batch 2 Awassi breed females in the second lactation = 100 heads) was carried out between May and August, thus carrying out 4 official controls.

During the milking period, only 4 checks were carried out during 2022, as the climatic conditions (excessive drought) reduced the milking period of the sheep, they were weaned at the end of August, so that at the beginning of September they were distributed to the mount. Thus, the milking period was carried out only for a period of 120 days.

The ewes were milked by hand, and the amount of milk milked from each ewe was determined using a precision electronic scale (± 5 g), by weighing the milking cups. The obtained data were then recorded in the control register and later used to calculate the amount of milk during the milking period.

The performance evaluation for milk production resulting from lactation analysis was based on applying the transformation coefficient in the suckling period and on the milking period exclusively on applying the alternative control method AT4. Estimation of the average total production of milk was carried out using the Fleischmann method (ICAR, 2018).

To calculate the quantitative production of milk over the entire lactation, the amount of suckled milk and that of milking milk were summed. The duration of lactation was 180 days (60 days suckling period and 120 days milking period).

Milk quality. Research on the quality of milk was carried out only during the milking period. For the analysis of milk quality, determinations were made regarding the chemical composition of milk in the main constituents (25 samples/control/lot), respectively dry substance, and within the dry substance the content of fat, protein, lactose and mineral salts. The determination of the chemical composition of the milk samples was carried out in SCDCOC Secuieni Bacău's own laboratory using the Funke Gerber type LactoStar milk analyzer.

Statistical analysis. The results obtained are presented as mean values ± standard errors of the mean. Microsoft Office Excel 2016 was used to calculate all statistical parameters (mean, standard deviation, coefficient of variation and standard error of the means) and the t-test (Student) to determine the significance of the difference between means (Raducuta et al., 2022). Differences were considered statistically significant at $P<0.05$ and indicated by specific superscripts.

RESULTS AND DISCUSSIONS

The assessment of body development in lambs during the suckling period represented a first objective of the research. The body development of the lambs in the first neonatal part is primarily dependent on how the gestation proceeded, with reference to ensuring the nutritional requirements specific to this period for the mother sheep.

Out of all the external factors, mothers' milk production most intensively influences the change in live weight in the first post-partum periods.

In order to correctly assess the dynamics of body development for the suckling period, individual weighings were carried out. In order to evaluate as well as possible the lactogenic capacity of mothers, weighings were also carried out at intermediate ages, respectively at 28 days and at weaning, respectively at 60 days.

The assessment of birth weight was determined by the individual weighing of lambs obtained during the lambing season held in 2022. The obtained lambs were weighed after the first 2 hours after lambing, a time interval in which the hair coat was ruffled and the lambs recovered.

The analysis of the live weight at 28 and 60 days respectively is extremely useful not only to be able to correctly analyze the growth intensity of the lambs in the first part of the lactation period, but also to be able to determine as precisely as possible the amount of milk consumed by to lambs during this time frame.

The birth weight of the lambs belonging to the F1 crossbreds was approximately equal to that of the Awassi breed lambs, being only approx. 1% lower (4.41 ± 0.066 versus 4.45 ± 0.084), the differences being insignificant (Table 1).

At the age of 28 days, the body weight of R1 lambs was 2.3% lower than that of Awassi lambs. The live weight determined at the age of 60 days was on average 14.37±0.151 kg in R1 crossbreeds obtained from backcrossing F1 crossbred females with rams from the Awassi breed and 14.81 ± 0.62 kg in the Awassi breed, the differences being only 3.1% (insignificant differences, $P>0.05$).

The evaluations based on the analysis of the data recorded on the occasion of weighing the lambs at birth, 28 days and at weaning (60 days), confirm that the rate of growth of the crossbreds lambs has approached that of the Awassi breed lambs, a fact that is confirmed by the existence of non-significant differences in all weights recorded during the suckling period (non-significant differences, $P>0.05$).

Evaluation of quantitative milk production during lactation. The estimation of the milk production for the suckling period was made by the method of the control weighings of the lambs made at lambing and at 60 days (at weaning), and based on these data the average amount of milk consumed by the lambs was calculated in the respective time interval.

This is necessary because in the first part of the nursing period the lambs accumulate body mass especially based on the intake brought by the consumption of mother's milk.

The estimation of milk production for the lactation period was made by the method of the coefficients of transformation of the increase gain made by the lambs into milk. Thus, in order to appreciate the amount of milk consumed by lambs in the first 60 days, it is considered that the lamb needs an amount of milk of 5.5 kg to achieve one kilogram of body weight gain in the period 0-28 days and 4.5 kg of milk for one kilogram of gain in the period 28-60 days. After the weaning period and based on the statistical processing of the values obtained as a result of the control weighing applied to the suckling (50 heads/lot), the total amount of milk consumed by the lambs during this time interval was determined.

Table 1. Evolution of body weight in lambs during the suckling period (n = 50 heads/lot) (kg)

Specification	Batch of crossbred lambs R1				Batch of Awassi lambs			
	$\overline{X} \pm s_{\overline{x}}$	V%	minimum	maximum	$\overline{X} \pm s_{\overline{x}}$	V%	minimum	maximum
Weight at birth	4.41±0.066	10.57	2.90	5.20	4.45±0.084	13.32	3.20	5.80
Weight at 28 days	8.69±0.091	7.38	7.15	10.00	8.89±0.152	12.08	7.00	11.30
ADG* 0- 28 days	0.153±0.003	15.79	0.088	0.196	0.159±0.004	16.79	0.114	0.225
Weight at 60 days	14.37±0.151	7.42	10.80	16.40	14.81±0.62	7.75	12.75	17.40
ADG 28- 60 days	0.182±0.004	14.51	0.114	0.251	0.185±0.004	16.39	0.128	0.291

*ADG - average daily gain

The data obtained, highlight the fact that Awassi sheep have a 4.4% higher lactation capacity compared to F1 crossbred sheep (51.91 ± 0.670 kg versus 49.72 ± 0.827 kg), the difference being significant (P <0.05). Worth noting that the R2 lambs had at the time of weaning an average body weight close to that of the Awassi breed lambs. However, the females of the Awassi breed have a significantly higher lactogenic potential (P<0.05) in the second lactation compared to that of the F1 crossbred sheep during the lambs' lactation period (Table 2).

Table 2. Milk production obtained during suckling period (60 days)
(n = 50 females and 50 lambs/lot) (kg)

Genotype	The milk quantity during suckling period					
	0-28 days		28-60 days		Total suckling period	
	$\overline{X} \pm s_{\overline{x}}$	V %	$\overline{X} \pm s_{\overline{x}}$	V %	$\overline{X} \pm s_{\overline{x}}$	V %
Batch of F1 ewes	23.53±0.525	15.79	26.19±0.537	14.51	49.72±0.827	11.76
Batch of Awassi ewes	24.45±0.581	16.78	27.47±0.636	16.39	51.91±0.670*	9.13

NS - non-significant differences (P>0.05); *significant differences (P<0.05); **distinctly significant differences (P<0.01); ***highly significant differences (P<0.001).

To determine the total milk production, the AT4 estimation method was used, performing four controls during lactation according to the methodology. On the control days, the amount of milk milked was determined alternatively, either at the morning milking or at the evening milking.

The method used to determine the amount of milk milked was gravimetric, using the electronic scale and standard milking cups. The interval between the control days was established in such a way as to comply with the official methodology recommended by ICAR.

After the end of the activities specific to the application of the last milk production control for the current year's lactation, the data were subjected to statistical processing. Based on the values obtained, it can be observed that there are no significant differences between the two batches (Table 3).

Table 3. Average daily milk production during the milking period (4 checks) (n = 100 ewes/batch) (g)

Specification	Batch of F1 ewes			Batch of Awassi ewes		Absolute and percentage difference (±; %)	
	n	$\overline{X} \pm s_{\overline{x}}$	V%	$\overline{X} \pm s_{\overline{x}}$	V%	±	%
Check I	100	1030.3±17.23	16.72	1065.92±15.83	14.85	35.62 NS	3.46
Check II	100	890.8±15.81	17.74	855.06±17.05	19.94	-35.74 NS	-4.01
Check III	100	718.15±17.83	24.83	726.94±20.02	27.54	8.79 NS	1.22
Check IV	100	458.21±15.49	33.81	499.61±11.71	44.73	41.4 NS	9.04
Average daily milk production	100	774.37±9.76	12.60	786.88±0.37	14.87	12.51 NS	1.62

NS - non-significant differences (P>0.05); *significant differences (P<0.05); **distinctly significant differences (P<0.01); ***highly significant differences (P<0.001).

The average daily milk production on the 4 controls for the F1 crossbred group was 774.37 ± 9.76 g with limits between 542.25 and 1006.5 g of milk, and for the Awassi breed group of 786.88 ± 0.37 g with limits between 489.75 and 1127.0 g of milk. The average level of daily milk production for Awassi sheep was about 1.62% higher than that of F1 crossbred sheep.

The differences between the controls are insignificant, including the average daily production during the milking period. The average milk production on the 4 controls for the Awassi ewes batch was 1.67% higher compared to the F1 crossbred females batch, the differences being insignificant (P>0.05).

The average level of milk production during the milking period for Awassi ewes was 1.59 kg higher than the F1 crossbred ewes. It can thus be said that the manifestation of the heterosis effect led to an increase in the milk production of the F1 crossbred sheep, the productive level being relatively similar to the Awassi breed. Compared to the initial production of Țurcană sheep during the milking period, the milk production of the F1 crossbred ewes in the second lactation during the milking period (120 days) is higher by 44.43 kg (approx. 88%), even under the conditions of drought manifested during the grazing period during 2022.

Over the entire lactation period (180 days) the F1 crossbred females recorded a total milk production of 144.85 kg, which is 2.61% lower than that of the Awassi sheep (148.63 kg)

Table 4. Milk production during the milking period (120 days) and during the lactation period (180 days) (n = 100 heads/batch) (kg)

Specification	Batch of F1 ewes			Batch of Awassi ewes		Absolute and percentage difference (±; %)	
	n	$\bar{X} \pm s_{\bar{x}}$	V%	$\bar{X} \pm s_{\bar{x}}$	V%	±	%
Check I	100	31.94±0.534	16.72	33.04±0.491	14.85	1,10^{NS}	3,44
Check II	100	26.72±0.474	17.74	25.65±0.511	19.94	-1,07^{NS}	-4,00
Check III	100	22.26±0.553	24.83	22.54±0.621	27.54	0,28^{NS}	1,26
Check IV	100	14.20±0.480	33.81	15.49±0.693	44.73	1,29^{NS}	9,08
Total milking milk	100	95.13±1.198	12.59	96.72±9.67	14.91	1,59^{NS}	1,67
Total milk (suckling milk+milking milk)	100	144.85		148.63		3.78	2.61

NS - non-significant differences (P>0.05); *significant differences (P<0.05); **distinctly significant differences (P<0.01); ***highly significant differences (P<0.001).

Evaluation of milk quality. The chemical composition of sheep's milk during the milking period is presented in Table 5. Thus, according to these results, it is found that milk obtained from Awassi breed sheep has a very significantly higher protein content (P<0.001), compared with that of F1 sheep. Also, the content of mineral salts and dry matter are significantly higher (P<0.05), compared to that of the crossbred F1 sheep (Table 5).

Table 5. Milk quality (%) (n = 25 milk samples/check/batch)

Specification	Batch of F1 ewes		Batch of Awassi ewes		Absolute and percentage difference (±; %)	
	$\bar{X} \pm s_{\bar{x}}$	V%	$\bar{X} \pm s_{\bar{x}}$	V%	±	%
Fat	7.54±0.22	14.66	7.60±0.11	7.41	0.06^{NS}	0.80
Protein	5.31±0.05	5.01	5.79±0.11	9.35	0.48***	9.04
Lactose	4.38±0.02	2.41	4.55±0.10	10.57	0.17^{NS}	3.88
Mineral salt	0.81±0.02	12.63	0.86±0.01	4.54	0.05*	6.17
Dry matter	18.05±0.23	6.35	18.80±0.26	6.87	0.75*	4.16
Density (g/cm^3)	1.0376±0.0001	0.022	1.0376±0.0002	0.022	-	-

NS - non-significant differences (P>0.05); *significant differences (P<0.05); **distinctly significant differences (P<0.01); ***highly significant differences (P<0.001).

Regarding the fat and lactose content, the differences between the two analyzed batches are insignificant ($P>0.05$). Also, the milk density is similar in the two batches of sheep. Overall, the values regarding the chemical composition of the milk recorded in the two batches of sheep fall within the limits quoted in the specialized literature (Tafta, 2008).

CONCLUSIONS

The females of the Awassi breed have a significantly higher lactogenic potential ($P<0.05$) in the second lactation compared to that of the F1 ewes during the lambs' lactation period;

The average daily milk production on the 4 controls for the F1 ewes group was 774.37 ± 9.76 g with limits between 542.25 and 1006.5 g of milk, and for the Awassi breed group of 786.88 ± 0.37 g with limits between 489.75 and 1127.0 g of milk. The differences in the milking period between the two batches are insignificant. Compared to the initial production of Țurcană sheep during the milking period, the milk production of the F1 crossbred females in the second lactation during the milking period (120 days) is higher by 44.43 kg (approx. 88%).

During the entire lactation period (180 days) the F1 crossbred females recorded a total milk production of 144.85 kg, which is 2.61% lower than that of the Awassi sheep (148.63 kg).

Compared to F1 crossbred sheep, milk from Awassi sheep has significantly higher protein content ($P<0.001$) and significantly higher mineral salts and dry matter content ($P<0.05$).

Research on the improvement of local sheep breeds by crossing with the Awassi breed will continue during 2023, especially since the results obtained in 2022 were affected by the drought factor.

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RESEARCH ON THE INFLUENCE OF TEMPERATURE AND HUMIDITY ON THE EX-SITU DEVELOPMENT OF QUEEN LARVAE

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Abstract

At it is known, the development of larvae from worker bees or queen bees is identical during the first two days of the larval stage. Morphological differences appear starting from the third day of larval development. The food administered to the larvae ex-situ, at regular intervals, forms a dry film upon contact with the larva, under conditions of temperature and humidity similar to those in the hive. The food drying phenomenon is due to the temperature-humidity variation, in the hive these parameters are regulated and monitored by the nurse bees. In the study, different combinations of temperature and humidity values in the controlled environment were tested, so as to increase the number of queens obtained, by increasing food intake. As a conclusion, at a temperature between 35-36℃ and a relative humidity between 75% and 85%, it is possible to obtain 20-25% queens that reach maturity from total number of two days old age larvae from the start of study.

Key words*: bees, controlled environment, ingestion, larvae, metamorphosis.*

INTRODUCTION

The experiences of growing bee larvae in the incubator, by administering diluted royal jelly, have been carried out in the last decades with quite good results (Human et al., 2006).
The problem to be solved is that of raising queens (queens) from worker larvae.
Most attempts have run into reduced larval growth due to reduced ingestion and controlled environmental factors, like temperature and humidity.
It is known that the larvae that develop into workers or queens are identical. Differentiation into the worker or queen bee occurs from the second day of larval life.
In the natural environment, in the colony of bees, during the first two days of the larva, they receive an identical food, consisting of royal jelly and honey. Starting from the second day, the worker larvae receive food consisting of royal jelly, diluted with honey in a large proportion.
It is obvious that the type of food decisively influences the development towards one or another type of bee. The queen larva receives the same type of food in the following days until the pupa stage. Basically, the queen larva floats on a layer of royal jelly.
Feeding is done by peristaltic, circular movements inside the cell. It is very important that the viscosity of the royal jelly allows these movements. The viscosity of the royal jelly must not increase beyond a certain limit because it makes it impossible for the larva to feed (Hanser, 1980).
In the natural environment, in the bee colony, the queen larva is fed small amounts of royal jelly, continuously administered by the nurse bees. The brood growth is directly influenced by the ability of the bee nurses to secrete royal jelly (Surlea (Surlea-Stoica) et al., 2022).
A queen larva is known to receive up to 1600 feeding visits.
The queen larva is fed *ad libitum*, the drying of the royal jelly being prevented by frequent feedings, up to the tipping phase.
In *ex situ* growth, these parameters are difficult to achieve (Tautz et al., 2003).

MATERIALS AND METHODS

Apis mellifera carpatica larvae were used in the experiment. The age of the larvae was precisely determined by the technique of isolation of the queen on the empty comb (Figure 1).

Figure 1. Two-day-old larvae after transvasation (Original photo)

All eggs laid between 10:00 and 12:00 are considered to be of the same age.
After 5 days - 3 days in the egg stage and 2 days in the larval stage, the larvae were transferred from the honeycomb to the 9 mm diameter grow-out beakers made of wax.
Previous experiments have shown that the diameter of the beaks does not influence the differentiation of larvae into worker or queen bees.
The space for the ex situ growth of the larvae was an oven with controlled temperatures and humidity, of our own construction.
The oven was equipped with a source of heat generation with a thermostat with a degree of variation of ± 0.1ºC and a fan to recirculate the air inside.
The research was carried out using three variants of temperature (T) - humidity (UR) combinations, respectively:
- Variant 1 (V1): T = 35 ± 0.5ºC and RH = 75%;
- Variant 2 (V2): T = 35.5 ± 0.5ºC and RH = 80%;
- Variant 3 (V3): T = 36ºC and RH = 85%.

The feed used was royal jelly diluted with a nutritious, vitaminized solution.
Food was administered at 2-hour intervals, throughout the larval phase, with the help of a dosing pipette. Food drops were deposited on the side wall of the beaks, imitating the feeding of nurse bees.
In this way, no temperature differences are created between the food administered and that existing in the barrel.
For each variant, 50 two-day-old larvae were introduced into the oven.
The larvae were introduced when the temperature-humidity ratio stabilized at the values specific to each work variant.
The duration of the research was 21 days, starting from the premise that the queen completes her development in 16-17 days, and the worker bee in 21 days.

RESULTS AND DISCUSSIONS

Although incompletely understood, the growth mechanism of bee larvae to obtain viable ex situ queens is influenced by the temperature-humidity relationship (Stabentheiner et al., 2010).
The three experimental variants had different results as a result of the influence of temperature and humidity in the ex-situ development of queen larvae.
In Variant 1, in the first 2 days after the start of the experiment, 23 larvae died of starvation due to dehydration of the food.
Of those that remained alive, only 12 removed excrement and began weaving the cocoon.
Eight larvae passed the metamorphosis phase, but died in the imago phase - unpigmented bees.
During the study period, in Variant 2, out of 50 larvae, 7 larvae died of starvation, and 26 removed excreta and wove the cocoon. 18 larvae completed metamorphosis and entered the imago phase, and 15 larvae completed full development at 17 days of age.
Of these, 4 presented morphological characters intermediate between queen and bee, with predominantly queen morphological characters (weight, shape of the mandibles, shape of the metatarsus).
Variant 3 offered the best results, respectively 6 larvae died during the larval period, through

starvation; 44 larvae eliminated excrement, wove the cocoon and completed metamorphosis; 27 larvae completed the imago phase and full development. Of these, 15 larvae completed full development in 17 days and 12 larvae in 16 ± 0.5 days.

As a result of the research carried out, three factors must be controlled for the success of ex situ rearing of queen larvae: food, temperature and humidity.

As for food, it has been found that the administration of fresh royal jelly at regular intervals is not a solution, as it dehydrates very quickly, leading to the death of the larva by starvation (Hanser, 1980).

Maintaining the growth temperature at 35 ± 0.5ºC as is done in the bee family, ensures larval growth up to day 3 - 4 larva, followed by high mortality (80% of larvae).

Keeping the humidity at around 75%, similar to the conditions in the bee family, did not influence the transition to the metamorphosis phase.

The stages and periods of development of queen larvae until the emergence of viable individuals are as follows:

1. Egg stage - 3 days.
2. The larval stage - 4.5 days.
3. Extended larval stage - 2 days.
4. Metamorphosis 1-2 hours.
5. Imago stage and pigmentation, full development - 7 days.

According to the centralized results presented in Table 1, Variant 3 provided the best results; 54% of the hatched larvae completed development, but not all had queen morphological characters. Only individuals that finish development in 16-16.5 days are considered to be queens.

Table 1. Results obtained in the larval growth variants

Variant/ Larvae number	Development stage				
	1	2	3	4	5
V1	50	27	12	8	0
V2	50	43	26	18	15
V3	50	44	44	44	27

Regarding the viability of the larvae, variant 3 also proved to be the best, offering temperature and humidity conditions favorable to the growth and development of the larvae (Figure 2).

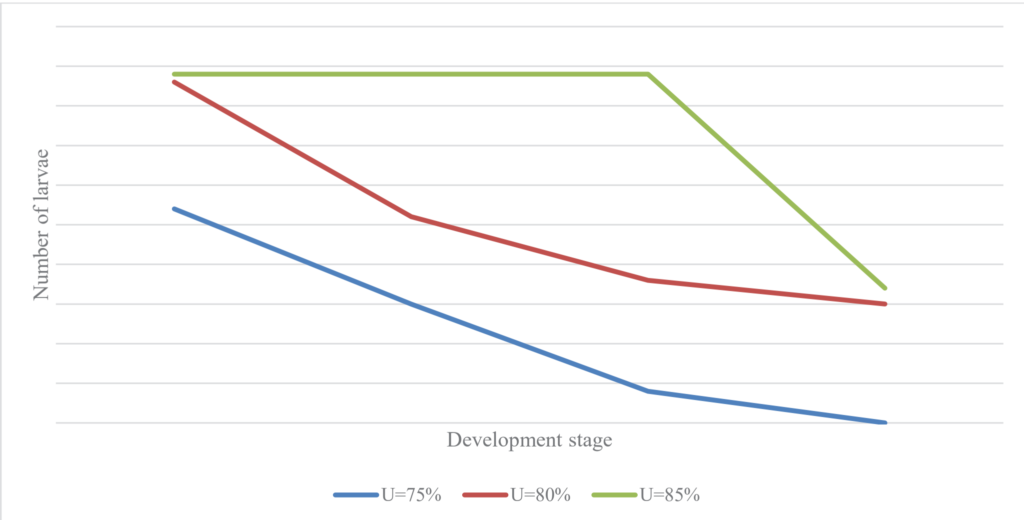

Figure 2. Larvae viability in the 3 experimental variants

Through the research carried out, an attempt was made to establish what are the suitable conditions for the ex situ growth of queen larvae.

The use of temperature and humidity parameter values in *ex situ* growth, similar to those of the bee family, (T =35 ± 0.5ºC and RH = 75%), in V1, does not lead to obtaining individuals with

specific queen characteristics. The larvae die in the various stages of development, through starvation, due to the dehydration of the food and the impossibility of ingesting it.
The viscosity of the feed is high and prevents the peristaltic movements of the larvae. They can no longer feed themselves or ingest a small amount of food.
Under natural conditions, the queen larvae are fed at short intervals, continuously, in the form of a drop of royal jelly and honey, deposited on the wall of the snout very close to the larva.

Figure 3. Larva during feeding (Original photo)

Larvae feed for long periods (Figure 3), interrupted by short periods of inactivity when molting occurs.
These periods of inactivity induce dehydration of the food around them, a phenomenon counteracted by nurse bees by searching for the larva and depositing fresh, excess food.
It is very important for the larva to ingest as much food as possible, until the excrement phase, when it stops feeding, in order to ensure the protein and energy necessary to complete metamorphosis and complete development.
The metamorphosis of bee larvae occurs with a high consumption of protein and energy, when a restructuring of the body is practically carried out (Figure 4).
The use in ex situ growth of royal jelly harvested from queen buds is not a solution because it dehydrates very quickly. Diluting the royal jelly, using different recipes, causes the larva to ingest food that is poorer in components that ensure development.

In Variant 1, the larvae die in various stages of development, by starvation, due to the dehydration of the food.
Variants 2 and 3 highlight the importance of the temperature-humidity relationship that influences larval development.
Temperature variations of ± 0.5°C, measured during the experiments, did not influence the development.

Figure 4. Larvae after metamorphosis (Original photo)

The lowest degree of dehydration of the food was achieved at the value of relative humidity of 85%, in the larval phase, until the elimination of excrement.

Figure 5. Queen with development completed in 16.5 days (Original photo)

After removing excrement and weaving the cocoon, the humidity value can drop to 75%, without influencing the phase of metamorphosis and imago.
Organisms that have completed their development as close as possible to 16 days after laying the egg (natural queen development cycle), have characters similar to queens (Figure 5).

Figure 6. Metatarsus from a queen with intermediate characters (Original photo)

Organisms that exceed this term also have intermediate characters between the queen and the worker bee (shape of the mandible, shape of the metatarsus, shape of the thorax, etc.) (Figures 6 and 7).

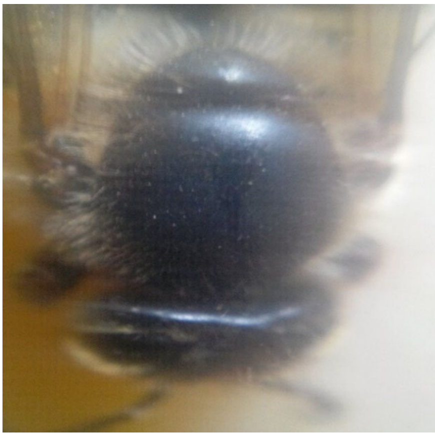

Figure 7. Queen-specific thorax (Original photo)

In organisms that finished development in 16.5 days, the lack of claws on one or more legs was observed (Figure 8), a phenomenon that also manifests itself in queens raised in bee families during periods of poor picking (Chuda-Mickiewicz & Samborski, 2015).

Figure 8. Clawless intermediate form (Original Photo)

Queens obtained in ex situ rearing were introduced into mating nuclei.
Out of 7 queens, 4 mated naturally and started laying eggs. The performances of the bee families, in which these queens were introduced, were similar to the apiary average, but no queen lasted more than one season.

CONCLUSIONS

The best results were obtained when the growth temperature was 36ºC and the relative humidity was 85%, resulting in 12 queens with a development period of 16-16.5 days and morphological characters, predominantly queen, about 24% of the larvae. These temperature and humidity values, correlated with the feeding of royal jelly diluted in various proportions with nutrient solutions, can increase the number of queens obtained *ex situ*.

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STUDY REGARDING THE BEEF MEAT PRODUCTION EVOLUTION WORLDWIDE AND NATIONAL LEVEL

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Abstract

The global beef industry has been growing steadily in recent years due to the fact that the largest beef exporters and importers in beef producing countries continue to invest financially in the global market. Beef, one of the most important products in the world livestock market, is often recognized as a premium source of protein in the human diet, therefore it is widely consumed in most countries around the world. In 2020, at the national level, the number of cattle slaughtered in industrial units (slaughterhouses) decreased by 15.6% compared to the previous year. Also, the beef production decreased from 196,000 tons in 2017 to 173,000 tons in 2020, even though the slaughter yield has increased. There are about 1 billion beef cattle worldwide, and compared to poultry and pigs, beef cattle have the lowest feed-to-meat conversion efficiency. Strategies to improve beef cattle performance emphasize operational and reproductive management, host genetics, functional efficiency of the rumen and respiratory microbiome, and forage structure and composition. There have also to consider the herd health and immunity and the need for beef cattle to thrive in a changing environment.

Key words: *cattle, consumption, meat; production; nutritive value.*

INTRODUCTION

The meat industry represents one of the most important sectors in the Romanian, national and world economy. An appropriate development of the food industry, correlated with the optimal use of available agricultural resources, may reduce the domestic market's dependence on imports and to ensure the nation's food security (Stanciu, 2014).

Beef represents a food source for population, being an essential social product in the rational human diet, reflecting the living standard of the population, respectively its quality of life. The social importance of beef is given by its high biological value and due to the fact that it is suitable to the processing of a wide range of prepared and semi-prepared products.

Beef contributes to the structure of food availability with 14.2% protein and 9.6% calories (Cziszter, 1999). Of the total meat production, the beef share is in average 33%, with an obvious decrease rate in recent years (about 0.89% annually) (Georgescu et al., 2000).

On average, beef contains 68.1% water, 20% protein, 10.8% lipids, 1.1% mineral salts and 183 calories per 100 g.

Beef is obtained from the cattle growth and fattening, being a source of profit, which does the agriculture more efficient through a higher utilisation of cheap fodder resources, such as: natural meadows, coarse fodder, industrial residues, etc., which are transformed into meat, a product with high biological and nutritional value.

In Romania, for this species, meat production is ensured from reformed adult and semi-adult bulls, but especially from young, fattened males and a small share from half breed, respectively meat breeds (Maciuc et al., 2019; Liciu, 1999; www.insse.ro).

Table 1 The chemical meat composition depending on the fattening level

Species	Fattening level	Body parts	Water %	Protein	Fat	Ash	Water/ protein ratio	Calories
Cattle	Thin	Shank	74.80	20.80	4..00	1.00	3.56	120
	Medium	Shank	66.40	20.00	8.00	0.90	3.32	160
	Fat	Shank	60.00	17.60	16.00	0.80	3.40	216
	Thin	Round	71.00	19.70	8.00	1.00	3.60	152
	Medium	Round	67.00	19.30	13.00	1.00	3.48	105
	Fat	Round	63.00	18.37	17.00	0.90	3.43	226
	Thin	Ribs	64.00	18.60	16.00	1.00	3.43	218
	Medium	Ribs	57.00	16.90	25.00	0.80	3.37	292
	Fat	Ribs	53.00	15.60	31.00	0.80	3.40	341

Source: Banu et al. (1996); Motoc (1986)

Even though there were imports of cattle breeds specialised in meat production: Aberdeen Angus, Charolaise, Limousine; Blonde d'Aquitaine, Hereford, Galloway; Highland; Aubrac; Romanian spotted - SIM etc., the total number of cattle is still reduced, and Romania, as an exporting country in the past, ended up importing beef (Pesonen et al., 2012; Holtcamp et al., 2019; Jiu et al., 2020).

Considering the above, and the accentuated decrease in herds and meat production, the malfunctioning of the reproduction system, the lack of a strong meat-producing sector - the commodity and not ultimately, the lack of assortment varieties of beef products (Stanciu, 2015), there was the need to do this research.

MATERIALS AND METHODS

In order to carry out the study, it was carried out a bibliographic documentation of cattle herds for meat on a global, European and national level for the recent years.

The data thus obtained were systematized, processed and interpreted through the methods specific to such research. The statistics, respectively the parameters, which characterize a normal distribution, are on the one hand the mean or median, and on the other hand the dispersion indices represented by the variance and the standard deviation of the observed character. For this purpose, the computer program S.A.V.C. was used. (Statistics Analysis of Variance and Covariance, 2003) to determine the arithmetic mean ($\overline{X}$), the error of the arithmetic mean ($\pm s_{\bar{x}}$) the standard deviation (s), the coefficient of variation (V %), and for the ANOVA significance tests respectively p, we used the computer program SPSS16.

RESULTS AND DISCUSSIONS

The aim of this research was to create an updated situation regarding the production of beef at the global, European and national level.

Cattle herds are unevenly distributed across the globe and continents. Analysing the distribution of livestock in 2021 (according to FAO), may be noticed that worldwide there are over 1.5 billion heads, of which only 5% are found in the E.U. As seen in Figure 1, the larger cattle herds than in the EU are found in countries such as India, Brazil and the USA.

Analysing the global and regional beef production, it may be observed similar uneven distributions and specific productivity developments, with the hierarchical order by country and region being slightly modified. Thus, of the 72,446,078 tons of beef obtained worldwide in 2021, most of 18% was produced in the USA (12,733,643 tons), followed by Brazil with a share of 13% (9,750,000 tons). Comparatively, 9% of the total beef production (6,882,070 tons) was achieved in the EU. The evolution of beef production expressed in tons worldwide during the period 2016- 2021 has an upward trend, which is different from the dynamics in the EU where things evolve downward (Figure 2).

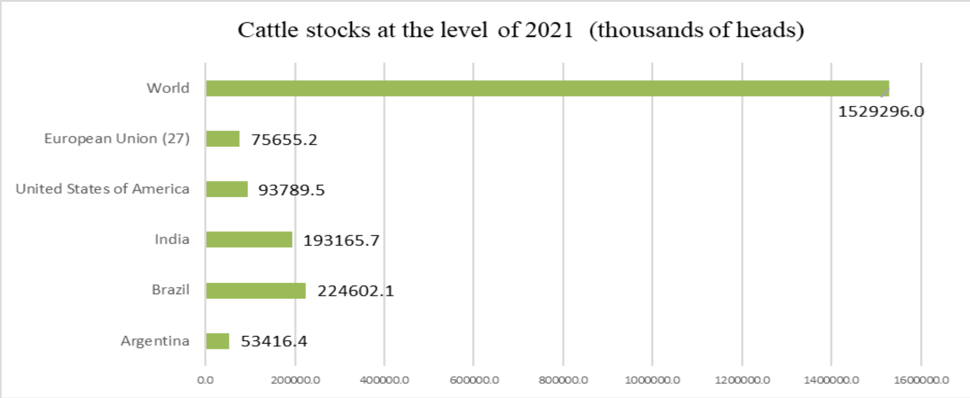

Figure 1. Cattle stocks in 2021 (thousand heads) (Source FAO)

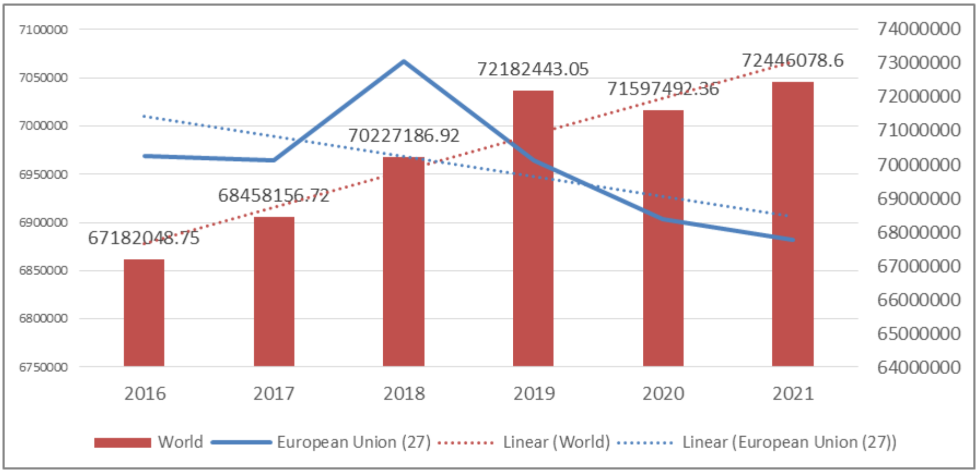

Figure 2. Evolution of beef production in the period 2016-2021 (thousands of tons) (source: FAO.org.)

In the E.U. beef was mainly obtained from slaughtered cows and young bulls, from herds specialised for beef production. Analyzing the cattle herds registered in the E.U. may be remarked that the countries with the largest numbers are France and Germany (Table 2).

Table 2 The evolution of cattle number in E.U. (27) during 2016-2022 (thousand heads)

YEARS	2016	2017	2018	2019	2020	2021	2022
European Union - 27 countries (from 2020)	79,697.54	79,009.94	77,840.10	77,161.16	76,551.10	75,705.30	74,855.71
Germany	12,466.59	12,281.20	11,949.09	11,639.53	11,301.86	11,039.66	10,996.96
France	19,373.38	18,953.58	18,613.04	18,172.97	17,815.67	17,330.08	16,986.19
Hungary	852.00	870.00	885.00	909.00	932.90	909.90	894.00
Poland	5,970.20	6,035.70	6,183.30	6,261.60	6,278.90	6,378.70	6,448.29
Romania	2,049.70	2,011.10	1,977.20	1,923.30	1,875.20	1,826.80	1,825.10

Source: EUROSTAT

In 2022, may be noticed that from 74,856 thousand animals, 22% were distributed in France (16,986 thousand heads), respectively 14.7% in Germany (10,997 thousand heads).

In the Eastern EU27 countries, the share of this activity is lower, and among them Poland occupies a more important role (8.6%), while Romania contributes only with 2.4%. From these effectives, on average in 2022, at the EU level intended for meat consumption were only 30%, respectively 22,662 thousand cattle.

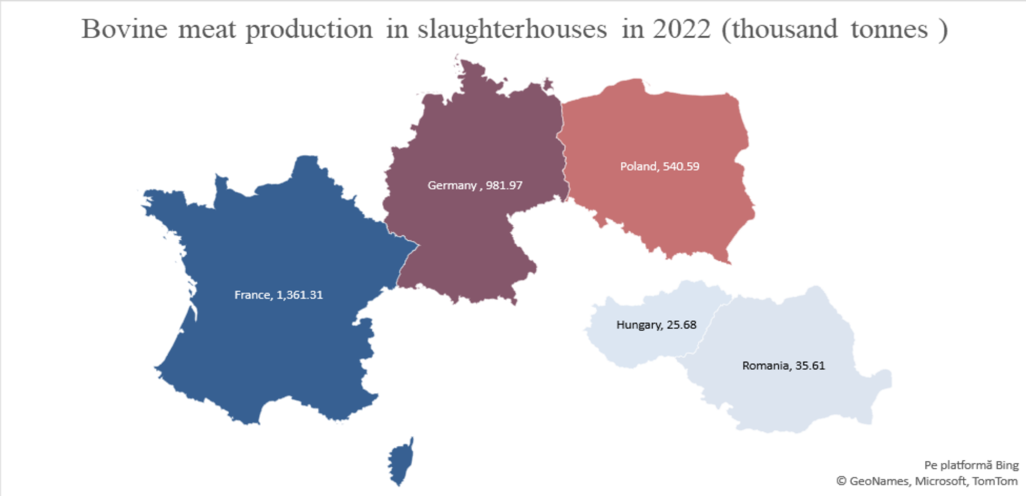

Figure 3. The distribution of bovine meat production in slaughterhouses for UE 27 in 2022 (thousand tonnes) (source: EUROSTAT)

The meat quantity resulting from the cattle slaughter in slaughterhouses for the period 2013-2022 to the EU level had a complex, winding evolution, with a general trend of growth until 2017, followed by a decrease process until the end of the analysed period. This trend differed across countries. Thus, in Germany the process was more accentuated, respectively by 15%, Hungary by 8%, Poland by 3%, respectively Romania by 40% for the same period. (Table 3 and Figure 3).

Table 3 Distribution of beef production to EU27 level (2016-2022, thousand tons)

	2016	2017	2018	2019	2020	2021	2022
European Union - 27 countries	6,888.36	6,898.10	7,008.99	6,907.99	6,822.30	6,801.91	6,636.95
Germany	1,148.00	1,124.00	1,102.00	1,106.00	1,090.00	1,072.02	981.97
France	1,464.15	1,442.18	1,460.00	1,428.46	1,434.59	1,424.32	1,361.31
Hungary	28.07	27.21	29.15	29.73	28.07	28.93	25.68
Poland	501.46	558.58	564.72	560.45	559.38	555.12	540.59
Romania	57.53	59.14	49.92	43.54	32.19	36.20	35.61

To the EU level there is an increase determined by a higher slaughter body mass and of the slaughter yield, starting with 2020, which is influenced by the productive performances' improvement and a continuous improvement of maintenance and nutrition technologies that are specific to the targeted category. It can be seen that the growth rate and direction vary widely, from one country to another, with differences that reflect different growth, economic and social conditions, but may be observed that in 2022 the slaughter yield growth remained on a floating line in some countries, and in others such as Romania it decreased (Figure 4).

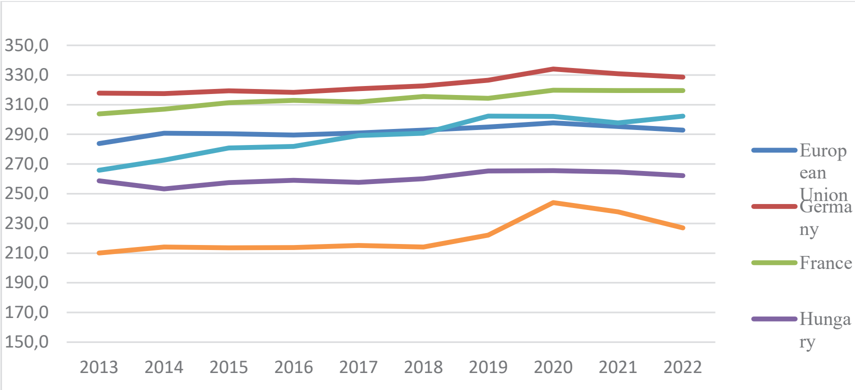

Figure 4. Evolution of the yield on slaughtered bovine carcass (source: EUROSTAT)

Romania, which has a high potential regarding grazing and raising beef cattle in extensive system, is ideally placed to meet the demands of the beef market. Romanian farmers have the ability to sustainably increase meat production in order to meet global demand. Aberdeen Angus cattle farms have the ability to produce quality meat, respecting the process of continuous welfare of the cattle, but also the environment.

Even though there have been recent increases in the beef price, also, are challenges for the meat industry, such as: production economics, changing regulations to the E.U. level, managing the perception of cattle health and welfare notions or the continuous protection of the environment.

If we refer to cattle herds, Romania is placed on the IX-th among the EU member states, after countries such as France, Germany, Spain, Ireland, Italy, Poland, Holland and Belgium. If we refer to the cattle density per 100 ha, Romania is placed on XX-th place, out of the 22 European countries, followed by Greece and Bulgaria. In Romania, the number of slaughtered cattle was decreasing throughout the period 2012-2021, the trend being accelerated after 2017. The same downward trend may be found in the North-East Region and South Muntenia Region (Figure 5).

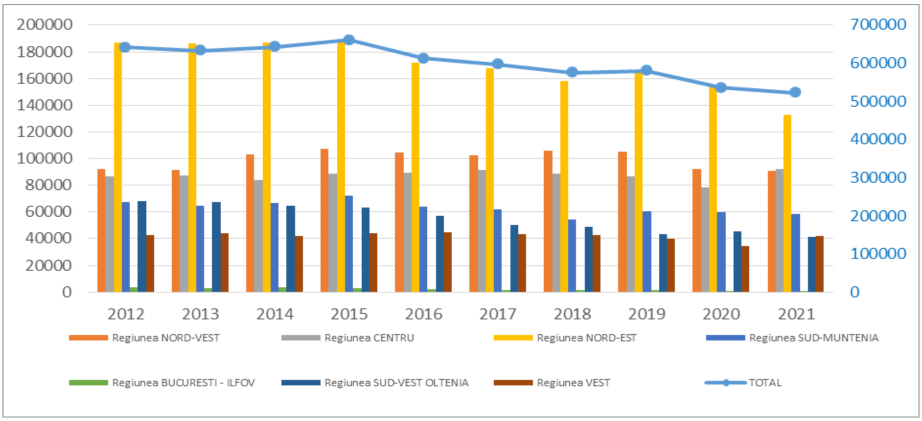

Figure 5. Total number of slaughtered cattle on development regions (2012-2021) (Source: INSSE)

According to INSSE data, in our country in 2021, cattle slaughtered in slaughterhouses were unevenly divided by region as follows: 44% of the total herd slaughtered was in the North East region, 21.62% in the South-Muntenia region, 13% in the Centre region 10%, and 11.28% in the North-West region (Figure 6).

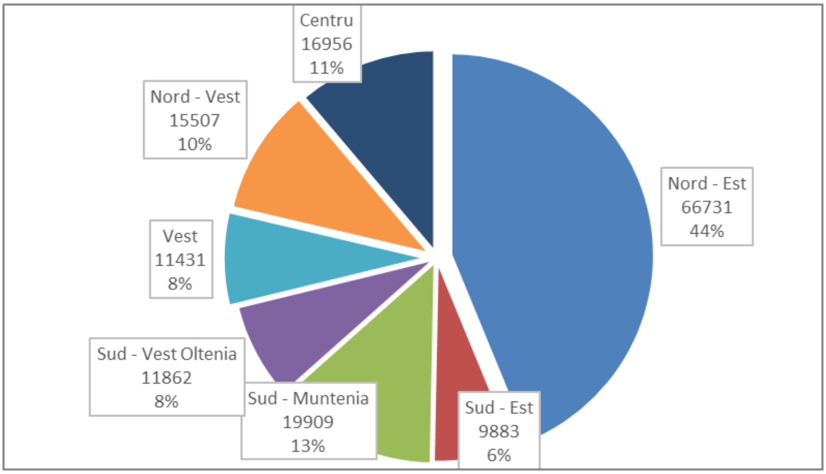

Figure 6. The cattle number slaughtered in Romanian slaughterhouses in 2021 (source: INSSE)

Regarding beef production, in 2022 totalised a 35,613 tons production, being down by 1.6% compared to 2021, when there was recorded a production of 36,198 tons of carcass, and the average weight of carcasses decreased by 5% in 2022 compared to 2021. Reported by region, in 2021 the largest share of production was in the North-East region (46%), followed by South Muntenia-(14%), respectively the Center (12%) (Figure 7).

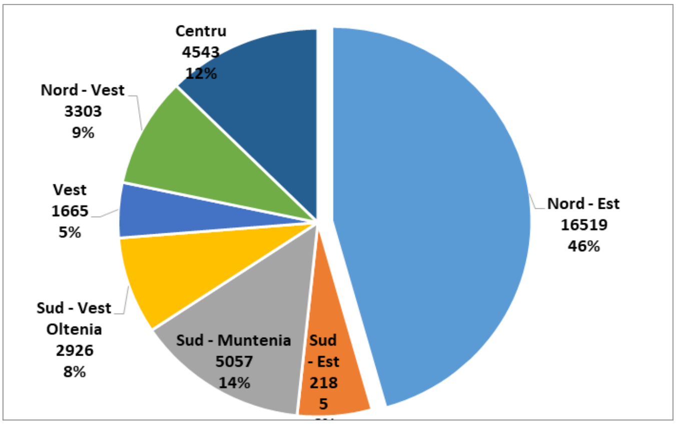

Figure 7. Beef cattle production obtained in slaughterhouses in Romania 2021(thousands of tons) (source: INSSE)

Table 4. Livestock dynamics, meat production and average carcass weight in the period 2021-2022 (source: INSSE)

	Slaughtered animals (thousands of heads) -		Carcass wight - tons		Average carcass weight	
	2021	2022	2021	2022	2021	2022
Total cattle livestock	523	503	85351	82618	163,2	164,3
Of which: in specialized industrial units (slaughterhouses)	152	157	36198	35613	238,1	226,8

CONCLUSIONS

Analysing in perspective the livestock and beef production evolution, may be observed that at the European level and even at the level of our country, the total production registers a slight decrease in the analysed period. This decrease will continue in the following decades, due to the influence of the increase of fodder and energy costs, following the war in Ukraine, which from 2021 has aggravated the problems for farmers around the world who were already struggling with drought and the reduction of land areas intended for grazing.

The limiting causes in the beef production increase are multiple, both at the European level but especially at the national level, the most important being:

- neglect of private owners of cattle raised for meat production;
- failure to ensure the fodder base, quantitatively and qualitatively;
- the reduced reproduction indices value to cows;
- the high morbidity and mortality rate in cattle subjected to fattening;
- reduced slaughter efficiency;
- the low level of weight gain, achieving, on average, at the national level, 60-66% of the average daily weight gain recorded in the EU countries.

The significant increase in fodder price, but also the change in food preferences of the modern people, influences meat production. In the coming years, beef will face a fierce competition from the pork and poultry market, which is considered as being tastier and obtained at lower costs.

Quality increasing of beef production will aim to maintain meat consumption at least at the current level. Improving the beef quality may be achieved by improving the juiciness, tenderness, aroma, colour and marbling of the meat, shelf life increasement of the products obtained after processing (hamburger), by feeding red beets to cattle (Marrone et al.., 2021).

Specialists recommend the expansion of industrial crossings between milk and meat breeds, the F1 crossbreeds being entirely intended for fattening and utilisation for meat. Increasing meat production is recommended through industrial crossings of milk breeds with bulls from meat breeds with hypermetric development (Charolaise, Blanc Belgian Blue, Blonde d'Aquitaine, Simmental and Limousine). The of ecological meat production intensification is achieved through the use of hypometric English breeds (Galloway and Highland) in crossbreeding.

The beef consumption increasement may be achieved by improving cattle fattening technologies, producing specialties, much better paid by consumers. In the future, production will be concentrated and specialised, by reducing the number of farms, but with an increase in the average herd raised on farm.

In the future, beef production will also belong to family farms, but the basis will be large, industrial-type production units. Regarding the meat production utilisation, it will be done in the form of prepared and semi-prepared products, in an integrated system.

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EFFECT OF USING *Lactobacillus plantarum*, *Bifidobacterium* AND ITS CONSORTIUM PROBIOTIC YOGURT IN PREVENTING THE GROWTH OF *Klebsiella pneumoniae* THAT CAUSES PNEUMONIA

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Abstract

Klebsiella pneumoniae has been linked with dysbiosis in the intestinal microbiota. This has led to the idea of improving respiratory defense by regulating the intestinal microbiota with the supplementation of beneficial strains, such as yogurt probiotics. To develop probiotics in yogurt, the addition of lactic acid bacteria (LAB) in it such as Lactobacillus plantarum and Bifidobacterium is needed. Yogurt is expected to improve human health, specifically in gastrointestinal and respiratory health. This study aims to study the effect of probiotic yogurt with a specific consortium based on Lactobacillus plantarum and Bifidobacterium to inhibit the growth of Klebsiella pneumoniae which causes pneumonia. The literature research method used Google Scholar and PubMed from 2000 to 2022 which obtained 60 journals. Based on observations, it was shown that Lactobacillus plantarum and Bifidobacterium were able to inhibit the growth of Klebsiella pneumoniae due to the decrease of pulmonary inflammation response after giving Lactobacillus plantarum, and the increase of IL-10 production by Bifidobacterium bacteria. Therefore, yogurt probiotics consortium could be used to prevent Klebsiella pneumoniae which causes pneumonia and lung damage.

Key words: *Bifidobacterium, consortium microbiota, Lactobacillus plantarum, Klebsiella pneumoniae, yoghurt probiotic.*

INTRODUCTION

Klebsiella pneumoniae (KP) belongs to a group of Gram-negative bacteria that are rod-shaped. KP is able to ferment lactose, and this bacterium is facultative anaerobic, causes pneumonia, and causes high morbidity and mortality (Mizgerd, 2006). When it develops into pneumonia, the immune system responds more severely, producing more pro-inflammatory cytokines, infiltrating neutrophils and macrophages, and causing massive lung damage (Soares et al., 2006; Zhang et al., 2000). Although localized inflammation is known to be protective against pathogen infection, as it prevents pathogen spread, unresolved hyperinflammation is associated with long-term inflammatory disorders and death (Medzhitov, 2008). Klebsiella infection is acknowledged as a major health concern due to increasing antibiotic resistance. Strategies to strengthen the immune response and develop pulmonary defenses are needed to overcome pulmonary infections. In this case, probiotics have been the leading candidate in this context. Probiotics are populations of live non-pathogenic microorganisms that are able to favorably impact the host if administered in sufficient and appropriate amounts. This group of beneficial bacteria is able to buffer host immunity against pathogenic bacteria in the gut and prevent risks (Reid et al., 2003; Adriani et al., 2021). Previous studies have shown that probiotics are effective in stimulating the immune system and strengthening non-specific immunity (Adriani et al., 2022). Although previous studies have mostly reported probiotic research focusing on intestinal mucosal immunity, one of the most essential effects of probiotics is to increase the host's mucosal immunity (Vieira et al., 2013). Studies of microbial-based therapy strategies to treat lung infections continue to grow increasingly popular. Between the probiotic effects, the addition of probiotics has been linked to a reduced risk of pneumonia, shorten the period of common cold, and protect the respiratory from any pathogens, including viral infections (Kawase et al., 2010; Kawahara et al., 2015; Song et al., 2014).

Recently, the bacterium *Lactobacillus plantarum* and Bifidobacterium have been studied for their potential as a starter for making yogurt products. *Lactobacillus plantarum* bacteria is a gram-positive bacterium commonly used in dairy products, meat, and various fermented vegetables. Based on research by Li et al. (2017), states that several bacterial strains based in Sichuan and Mongolia show high coagulation abilities and proteolytic activity. These bacteria can survive well in fermented and post-fermented milk. This shows that milk media is a suitable medium for the growth of *Lactobacillus plantarum* bacteria.

Lactobacillus plantarum and *Bifidobacterium* are several lactic acid bacteria that also act as a probiotic, a live bacterium that can provide health effects to the host if consumed in sufficient quantities (FAO & WHO, 2006). Probiotics are included in functional foods because probiotics can maintain a balance in the intestinal microbiota, increase immunity, and other benefits for health (Quigley, 2019; Adriani et al., 2020). If this bacterium is used as a starter in the yogurt products, then the product is included in one of the functional food products. Therefore, research on the literature review of the potential of probiotic bacteria *Lactobacillus plantarum* as a starter in the production of functional yogurt needs to be carried out to provide an overview, information, and ideas from various previous studies. The results of this literature review are expected to be used as a reference for further research.

MATERIALS AND METHODS

The literature research used Google Scholar and PubMed from 2000 to 2022, which obtained 60 journals. Based on the observations, it was shown that *Lactobacillus plantarum* and *Bifidobacterium* were able to inhibit the growth of *Klebsiella pneumoniae* in the lungs. The probiotic works against the pathogenic bacteria were indicated by the decrease of pulmonary inflammation response after giving *Lactobacillus plantarum*, and the increase of IL-10 production by *Bifidobacterium* bacteria.

RESULTS AND DISCUSSIONS

Pneumonia is becoming a serious global health problem. Respiratory tract infections, especially pneumonia, have been a major cause of morbidity and mortality over the past 50 years; therefore, it continues to be a major concern in medical research (Armstrong et al., 1999).

The potential of probiotics for infectious diseases and pulmonary infections has been of great interest as awareness of the role of the immune system through gut mycoflora has increased (Forsythe, 2014).

The main mechanism of probiotics is to prevent microbial adhesion and exclusion competition of harmful microorganisms by increasing intestinal mucosal adhesion, synthesizing antimicrobial proteins, maintaining and strengthening epithelial barriers, and modulating the immune system (Bermudez-Brito et al., 2012; Adriani, Rifki & Widjastuti, 2020). The process can be seen in Figure 1.

Lactic acid is produced by LAB group microorganisms from various carbon sources, including simple carbohydrates (Carr et al., 2002). These beneficial microorganisms produce secondary bioactive metabolites, especially enzymes that are able to inhibit and stop the growth of microbes that are not beneficial to health, besides that they also produce exopolysaccharides and bacteriocin. These elements are connected to various probiotic effect mechanisms (Leroy & De Vuyst, 2004; de Melo Pereira et al., 2018; Lesmana et al., 2021). This mechanism for repairing the digestive tract is well known.

The interest in the application of these microbes to the prevention of lung infections is well demonstrated through the utilization of *Bifidobacterium* in cases of respiratory infections, such as asthma, allergies, and even influenza infections (Kawahara et al., 2015; Verheijden et al., 2015; Drago et al., 2015).

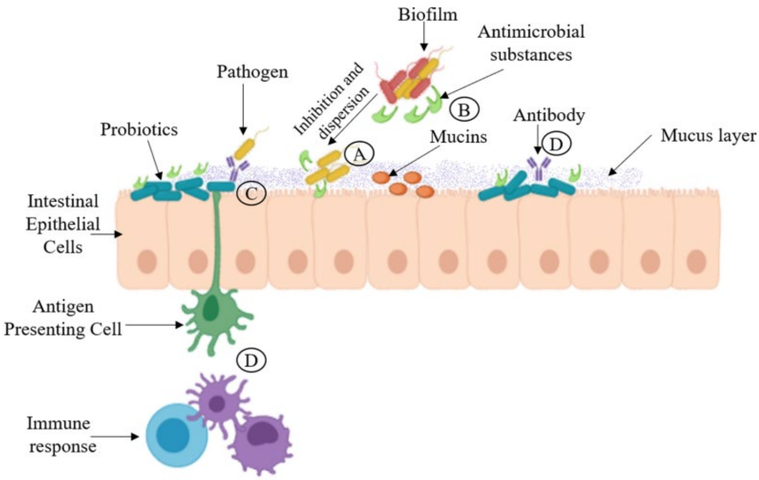

Figure 1. Mechanisms of how probiotics work: (A) Competitive exclusion of harmful bacteria. (B) Production of antimicrobial agents. (C) Enhanced adhesion to the intestinal mucosa and increased the epithelial barrier. (D) Stimulation of the immune system (Silva et al., 2020).

Therefore, maintaining immunity to the respiratory organs is important in reducing cases of pathogenic infections and also preventing them. Interestingly, the effects of diet or antibiotic treatment on the gut microbiota are associated with lung disease (Kim et al., 2014; Russell et al., 2012; Thornburn et al., 2014). Recent studies, as reported by Trompette et al. (2014) in mice, it appears that the metabolites produced by the fermentation of dietary fiber by intestinal microbes are able to protect the lungs from infection with pathogenic bacteria. *Bifidobacterium* bacteria are the largest short-chain fatty acid-producing bacteria in the fermentative commensal bacteria group (Fukuda et al., 2011). Several recent reports justify that the most abundant short chain fatty acid in the intestine is acetate. It was also shown that acetate is able to regulate inflammation prevention through various signaling pathways (Arpaira et al., 2013; Maslowski et al., 2009).

A study of the effects of the probiotic *Bifidobacterium* by mice which has been reported by Vieira et al. (2016) showed that the application of this probiotic was able to protect sample animals from *Klebsiella pneumonia*, indicated by a decrease in inflammation with increased production of IL-10, in addition to significant protection against the lungs, clearly indicated by a decrease in the population of pathogenic bacteria. One of the main reasons for this effectiveness is the increased activation of the TLRs Mal adapter protein. IL-10 can have various influence, depends on context and timing. In the setting of a lethal dose of *Klebsiella*, systemic inhibition of IL-10 enhanced bacterial clearance and reduced mortality (Lenz et al., 2007). IL-10 was also shown to prevent immunopathological consequences from infection caused by various pathogens (Wilson et al., 2005; Redford, Murray & O'Garra, 2011; Loebbermann et al., 2012). IL-10 is an important cytokine that plays a role to reduce inflammation (Serhan et al., 2007). Beside on this, the higher levels of IL-10 secreted by *Bifidobacterium* bacteria treatment in the lung to treat *Klebsiella* infection plays a role in the resolution of inflammation and restoring tissue homeostasis.

Lactobacillus spp. also has been largely recognized for having an anti-pathogen effect that is safe to consume (Ripamonti et al., 2011). However, the exact mechanisms of *Lactobacillus* spp. effect are not yet widely understood. A study by Yan et al. (2021) stated that *Lactobacillus plantarum* can improve mammals' digestive tract condition by increasing intestinal bacteria that utilize fatty acids such as Bacteroidetes and Blautia. To eliminate the strong *Klebsiella pneumoniae* and killing effect of Bacteroidetes and *Lactobacillus plantarum*, when *Lactobacillus* *plantarum* secretes a high amount of acetic acid, it can acidify the intracellular environment of *Klebsiella* and prevent its growth.

In addition, previous epidemiological studies showed that *Klebsiella pneumoniae* bacteria are liable for nosocomial infections that came from the gastrointestinal reservoir of the patients (Podschun & Ullmann, 1998). Hence, the administration of probiotics could be an alternative to prevent or even treat the respiratory infections caused by *Klebsiella pneumoniae*. These bacteria would not only prevent *Klebsiella pneumoniae* from proliferating in the intestine, but they would also control the inflammatory response and boost lung immunity.

Further research by Vareille-Delarbre et al. (2019) investigated the effect of *Lactobacillus plantarum* on the host immune response generated in lung tissue and cells by *Klebsiella pneumoniae*. Notably, *Klebsiella* did not affect the immune response in intestinal epithelial cell lines, in contrast to respiratory epithelial cells, possibly because the intestinal cells could handle the presence of *Klebsiella*. This is suitable with the fact that the human gastrointestinal tract is a reservoir for this pathogen and its colonization process does not lead to any harmful intestinal disorders (De Champs et al., 2004). *Lactobacillus plantarum* significantly decreased in the proinflammatory cytokine (KC, IL-6, and TNF-α) as the innate immune response from the pathogen. Although most studies have shown an impact of probiotics on the innate immune response (Giorgetti et al., 2015; Visozo et al., 2009; Boirivant & Strober, 2017), recent studies have speculated that these bacteria may also have an immunomodulatory effect at distal sites. (Vieira et al., 2016; Salva, Villena & Alvarez, 2010; Park et al., 2013; Racedo et al., 2006; Khailova et al., 2013).

Lactobacillus plantarum is also known to have the ability to secrete high amounts of IL-12. According to research by Vissers et al. (2010), all *Lactobacillus* strains were tested and stimulated by IL-10, TNF-α, and IFN-γ and IL-1β, the results of his research showed that *Lactobacillus* strains were able to act as strong inducers of the proinflammatory cytokine TNF-α (Timmerman et al., 2007; Maragkoudakis et al., 2006;) and also IL-12 (Haller et al., 2000; Shida et al., 2006), although other researchers reported different results that increased IL-12 production was not significant by lactic acid bacteria (Niers et al., 2005; Drouault-Holowacz et al., 2006). IL-12 production can be stimulated by all *Lactobacillus plantarum* strains, although Lactobacillus acidophilus cannot. The observed differences in IL-12 induction were due to differences in the cellular responses of these species and strains. These data indicate that *Lactobacillus plantarum* has a better ability to produce innate cytokines, such as IL-12 and TNF-α. *Lactobacillus plantarum* elicited higher production of IFN-γ, IL-12, and TNF-α, and therefore had a better capacity to enhance the response of the Th1 subset, compared to *Lactobacillus acidophilus*. In general, *Lactobacillus plantarum* strains are known to induce high amounts of IL-12, compared to *Lactobacillus acidophilus* (Vissers et al, 2010).

CONCLUSIONS

Klebsiella pneumoniae infection can be prevented by consuming probiotic yogurt containing *Lactobacillus plantarum* microbiota. Based on the trials conducted on mice, it can lessen the pulmonary inflammation, by decreasing numbers of lung innate immune cells (macrophages and neutrophils) as well as cytokines (keratinocyte-derived chemokine [KC], IL-6, and TNF-α) in the bronchoalveolar fluid, and generate an immunosuppressive Treg response in lungs.

While the consumption of *Bifidobacterium* leads to a faster resolution of inflammation associated with enhanced IL-10 production, and lessen the lung injury based on a result of a significant reduction of harmful bacteria that contributed to the mice mortality.

Therefore, based on the literature review, we can conclude that the combination of these two bacteria will greatly reduce and even maximize the prevention of *Klebsiella pneumoniae* infection.

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DEVELOPMENT OF A FUNCTIONAL MEAT PRODUCT WITH SEA BUCKTHORN OIL AND ANALYSIS OF ITS SENSORY AND PHYSICOCHEMICAL QUALITY

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Abstract

The present study was carried out to follow the technological manufacturing process of functional meat products - three batches of pork tenderloin injected with sea buckthorn oil in three different proportions of 1, 3, and 5% and to analyze their quality. The products were obtained in the Meat and Meat Products Microproduction Workshop of the University of Life Sciences Iasi. After the experimental batches were made, their sensory and physical-chemical analysis was carried out. The sensory analysis of the three types of muscle injected with sea buckthorn oil involved the application of a CATA questionnaire to a group of evaluators, which showed that these products have good consumer acceptability, especially the 3% and 1% sea buckthorn oil batches. The physicochemical aspects were analyzed in terms of color, pH, and raw chemical composition. The colorimetric analysis showed a decrease in the brightness of the samples with an increase in the amount of sea buckthorn oil in the batch. As for the chemical analysis, the increase in the amount of sea buckthorn oil resulted in very different values between batches.

***Key words**: functional food product, meat product, quality parameters, sea buckthorn oil.*

INTRODUCTION

In recent years, as people pay more and more attention to health, the physiological functions of food have received increasing attention (Arihara et al., 2006).
Meat is a good source of dietary protein and has a high biological value (Hathwar et al., 2011). However, consumers often label meat negatively, characterizing it as a high-fat, cancer-promoting food (Valsta et al., 2005). For these reasons, consuming meat and meat products is often avoided to minimize the risk of cancer, obesity, and other diseases. However, such a view ignores the essential importance of meat in maintaining human health. Although the importance of meat and meat products in consumer health has been widely discussed in scientific articles to better educate and inform consumers, data are still lacking in many countries (Arihara et al., 2004; Biesalski et al., 2005; Cassens, 1999; D'Amicis and Turrini, 2002; Desmond and Troy, 2004; Enser, 2000; Ferna´ndez-Gine´s et al., 2005; Garnier et al., 2003; Gregory, 2004; Higgs, 2000; Jime´nez-Colmenero et al., 2001, 2006; Kues and Niemann, 2004; Ovesen, 2004a, 2004b; Tarrant, 1998; Valsta et al., 2005; Verbeke et al., 1999).
One of the most maligned and avoided components of meat and meat products because of the risks it is thought to pose - fat - can be minimized by selecting lean cuts of meat, eliminating the fat, manipulating the diet to alter the fatty acid composition, and controlling portions appropriately to reduce fat intake and calorie intake. In recent years, particular attention has been paid to developing meat products with health benefits by introducing bioactive ingredients (Arihara et al., 2004).
Functional foods are an outstanding and promising category of foods that exhibit beneficial characteristics such as cholesterol-lowering properties, antioxidant, and anti-cancer properties that are considered quite attractive by consumers (Ioannis and Maria, 2005).
Increasing consumer demand for healthy food has initiated extensive research and development of new products in the food

industry. To meet this ever-increasing consumer demand, the food industry is reformulating food products to improve the physiological functionality of inherent nutrients or by adding a bioactive ingredient (Day et al., 2009).

One of the most interesting functional ingredients that can be added to meat products is sea buckthorn fruit oil, which contains high concentrations of vitamin C, carotenoids, tocopherols, and other bioactive compounds with a strong antioxidant role, in addition to the unique profile of beneficial lipids in the pulp and peel of the berries. Sea buckthorn oil contains a multitude of active substances. Scientific studies confirm the content of almost 200 ingredients that ensure the multidirectional effect of this type of vegetable oil (Zielińska and Nowak, 2014; Rajaram, 2014; Ng et al., 2014; Walczak-Zeidler et al., 2012; Kall io et al., 2012). The phenolic fraction is a major component in the bioactive function of sea buckthorn oil and has recently been studied for its possible health effects. According to Ma et al. (2016), one of the main aglycones in sea buckthorn is isorhamnetin. Isohamnetin, which is mainly found in the aqueous fraction of the fruit, has been shown to exhibit high antioxidant activity, even higher than that exerted by ascorbic acid, and this has been confirmed by various chemical tests (FRAP, DPPH), as reported by Pengfei et al. (2009). Nowadays, many food companies are striving for clean-label products, and the phenolic fraction in sea buckthorn is emerging as a possible natural antioxidant that can replace synthetic antioxidants. In addition, the phenolic fraction of sea buckthorn fruit has been shown to significantly decrease hydrogen peroxide-induced plasma peroxidation and increase clotting time in a test tube study, thus demonstrating interesting anticoagulant activity (Olas, 2018).

The trend in global food consumption away from traditional foods towards functional foods with guaranteed health effects will significantly increase the use of foods incorporated in meat products. Developing and marketing these products is a complex, costly, and risky task. The success of a product involves the combined efforts of diverse professional branches, notably nutritionists, epidemiologists, food technologists, natural product chemists, and others. Also, regulatory issues, sensory evaluation, and supermarket simulation are important aspects of the success of a new, functional meat product. Ultimately, the success or failure of functional foods depends on the characteristics of the product, its commercial viability, and, in turn, the nature, extent, and management of collaboration between related fields (Arihara et al., 2006).

The utility and ultimate applicability of functional materials or ingredients depend on four major factors - availability, durability, marketability, and consumer acceptance (Nimish et al., 2011).

The study aimed to manufacture functional meat products (3 batches of pork tenderloin injected with sea buckthorn oil in proportions of 1, 3, and 5%) and to analyze their sensory and physicochemical quality.

MATERIALS AND METHODS

The present study was carried out in the Meat and Meat Products Microproduction Workshop, in the Sensory Analysis Laboratory, and in the Meat and Meat Products Technology Laboratory; all part of the Faculty of Agriculture of the "Ion Ionescu de la Brad" University of Life Sciences Iasi.

The raw meat material used to make the 3 batches of pork tenderloin with sea buckthorn oil and the control batch was purchased from the local food market, as the sea buckthorn oil. The percentage values required to manufacture the batches presented in this paragraph are given in Table 1.

Table 1. Composition of the experimental batches

Batch	Ingredients (%)		
	Pork tenderloin	Seabuckthorn oil	Salt
L1SO1	97	1	2
L2SO3	95	3	2
L3SO5	93	5	2

The experimental protocol consisted of injecting pork muscle with three different concentrations of sea buckthorn oil (1, 3, and 5%), using a manual injection device. Before injection, the muscle was subjected to a dry salting process with a 2% salt concentration, lasting 24 h. The salted and injected tenderloin with sea buckthorn oil was inserted into a textile membrane with pepper, tied at one end, and hung on the raster trolley. After this step, thermal processing of the products followed, which included the steps and parameters shown in Table 2. After completion of the steps shown in Table 2, the products were cooled within 6 hours, packed, labeled, vacuum-packed, and stored at 2-4ºC.

Table 2. Thermal process stages and parameters for the pastrami

Stage	Time (minutes)	The temperature in the heat treatment cell (ºC)	The temperature in the thermal center of the product (ºC)	Humidity (%)
Drying	30	65	55	25
Smoking	30	65	55	25
Boiling	-	72	69	99
Drying	20	80	72	25

After obtaining the three batches of pork tenderloin with sea buckthorn oil, sensory and physicochemical analyses were carried out on them.

The sensory analysis of the three batches was carried out by a group of 39 students of the University of Life Sciences of Iasi in the Sensory Analysis Laboratory of the same University. The students who were part of this analysis were aged between 20-22 years. Four tasting sessions were carried out. At the beginning of these sessions, it was explained to the evaluators the content of the questionnaire, how to fill it in, and the actual tasting procedure. The samples from the three batches were sliced using a slicer to ensure uniformity between them and were coded with three random numbers. The evaluators were asked to tick the characteristics identified in the questionnaire in the samples, namely: crust integrity, noticeable sea buckthorn oil, heavy sea buckthorn oil in the mass of the product, uniform color, acid aroma, pepper aroma, characteristic meat flavor, soft sea buckthorn oil taste, salty taste, tasteless and balanced taste (meat and sea buckthorn oil). The results of the questionnaires were further processed in the XLSTAT software, using the CATA analysis contained therein.

The physical analyses applied to the products studied consisted of instrumental colorimetric analyses and instrumental pH analyses. The color analyses of the studied batches were carried out using the portable Konica Minolta Chroma Meter CR-410. The colorimetric analysis was performed through the CIELAB three-dimensional color system, which measured the quantitative relationship between the color parameters L* (black-white), a* (red-green), and b* (yellow-blue), with the D65 illuminant. The chronometer was calibrated before the analysis on a standard white calibration plate. A total of 5 section readings were taken for each batch.

Hanna Instruments portable pH meter for meat and meat products, model HI99163, was used to measure the pH value. Five measurements were made for each batch, each of which was carried out at a different location of the samples studied.

The determination of the chemical composition of the three batches of pork tenderloin with sea buckthorn oil consisted of measuring their main chemical components, represented by: protein, collagen, lipids, salt, and moisture. In order to carry out this type of analysis, the Food-Check automatic meat analyzer was used, which uses NIR (Near InfraRed) spectroscopy which is the analytical technique of using infrared radiation to determine the organic composition of the samples analyzed.

The physicochemical analyses were subjected to ANOVA and Tukey's test at a 5% level of significance ($p < 0.05$) for comparison of mean values.

RESULTS AND DISCUSSIONS

The results of the sensory analysis, using the CATA questionnaire, were evaluated using multiple factor analysis (MFA), as shown in Figure 1. Factors 1 and 2 show the variation that exists between the studied groups. In order to perform the multiple-factor analysis, an ideal was chosen, which has the following characteristics: crust integrity, uniform color, pepper aroma, characteristic meat flavor, soft sea buckthorn oil taste, noticeable sea buckthorn oil, balanced taste (meat and sea buckthorn oil). The similarity chart, which is divided into four quadrants, shows the attributes of the ideal product, but also those of an undesirable product: heavy sea buckthorn oil and intense sea buckthorn oil taste. Also, three other characteristics that were part of the questionnaire (acid aroma, salty taste, and tasteless) do not appear in the graphic, as they were not identified by the 39 evaluators in the three batches studied. By analyzing Figure 1 it can be noticed that the most desired characteristics are found in the two cadrans on the left side of the graph, and the batch closest to the ideal is L2SO3 (tenderloin injected with 3% sea buckthorn oil), which possesses the most desired sensory characteristics by consumers. Batch L1SO1 is also on the left side of the graph, which means that this batch also has sensory characteristics that are to consumers' liking. The last batch, L3SO5, is located on the right side of the graph, but not far to the left. This batch did not appeal to consumers in the same way as the other two batches, but it does not stray very far from them either. The main negative characteristic identified by a significant number of reviewers was the pronounced taste of sea buckthorn oil, which was not to their liking.

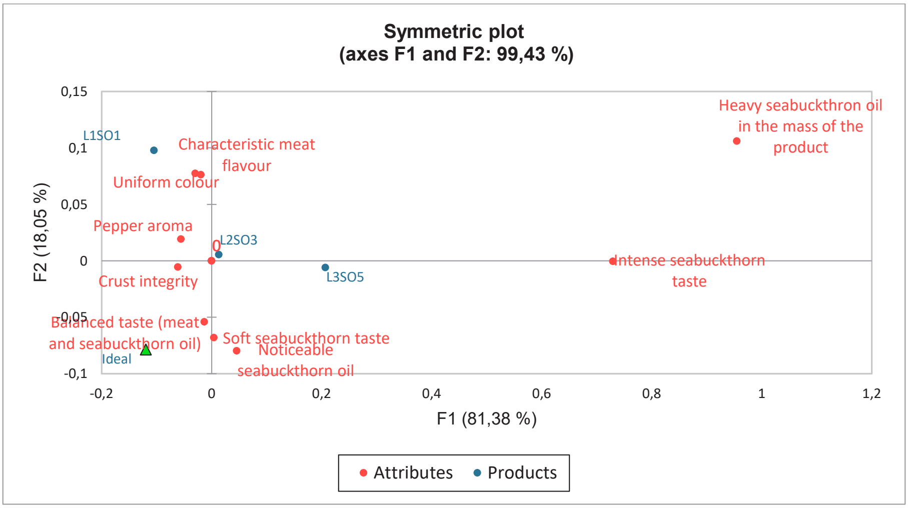

Figure 1. CATA analysis results for the three batches studied, using multiple factor analysis (MFA)

The color parameters (L*, a*, b*) are presented in Table 3 for the 3 batches studied. The L* parameter (0 - darkness; 100 - lightness) recorded the highest value in the L1SO1 batch, with the value of this parameter gradually decreasing with an increasing amount of sea buckthorn oil in the batch. In line with this statement, we can observe the value of L* for the L3SO5 batch (Table 3), which is significantly lower than the value of the L1SO1 batch ($p < 0.0001$). As for the parameter a* (negative - green color; positive - red color) we can observe that its value increases significantly with increasing the amount of sea buckthorn oil ($p < 0$). The highest mean value for the parameter a* is found in the L3SO5 batch, and the lowest in the L1SO1 batch (the batch with the lowest amount of sea buckthorn oil). As with the L* parameter, the L2SO3 batch also ranks with the a* parameter in the middle position between the other two studied batches. For the parameter b* (negative - blue color; positive - yellow color similar path can be observed as for a*, meaning that the mean value for b* increased with the increase in the oil content, but the differences are not significant ($p = 0.217$).

Table 3. Mean (± standard deviation) of colour parameters (L*, a*, b*) for the samples of tenderloins

Parameters	Batches			p-value
	L1SO1	L2SO3	L3SO5	
L*	56.92±0.316	51.16±1.099	50.99±0,647	<0.0001
a*	14.11±0.283	16.82±0.609	18.26±0.569	<0.0001
b*	14.19±0.941	15.16±1.920	18.068±1.563	0.217

ANOVA Tukey test: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1

Table 4 shows the physicochemical composition of the three batches of tenderloin injected with buckthorn oil. All parameters studied showed significant differences between batches ($p < 0.0001$), except for pH, which showed insignificant differences ($p = 0.184$). For protein content, the L2SO3 batch recorded the highest value, the second highest value in descending order was identified for the first batch (L1SO1), and the lowest value was found in the L3SO5 batch. After analyzing the mean values for protein content, we can conclude that the addition of sea buckthorn oil does not influence its value. The collagen content shows the same order as the protein in terms of mean values.

Lipid content within the three batches of tenderloin varied significantly ($p < 0.0001$), with the highest value found in batch three (the batch with the highest content of sea buckthorn oil - 5% and the lowest in batch L2SO3. Batch L2SO3 has a lower amount of fat than batch L1SO1, the reason for this value being the uneven distribution of the sea buckthorn oil throughout the tenderloin mass.

Table 4. Physico-chemical composition of the experimental batches

Studied characteristics	Experimental batches			p-value
	L1SO1	L2SO3	L3SO5	
Protein (%)	21.4±0	21.8±0.02	21.3±0	<0.0001
Collagen (%)	19.78±0.037	20.22±0.02	19.72±0.02	<0.0001
Fat (%)	4.14±0.024	2.38±0.058	4.58±0.02	<0.0001
Moisture (%)	74,08±0.037	75.52±0.058	73.64±0.04	<0.0001
Salt (%)	1.84±0.024	1.92±0.048	2.52±0.02	<0.0001
pH	6.18±0.010	6.19±0.005	6.17±0.007	0.184

ANOVA Tukey test: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1

The lowest moisture content was identified in batch L3SO5, this result could be due to the higher amount of sea buckthorn oil in this batch. For the L2SO3 batch, the average value of water content is the highest of all three studied batches, even though this batch does not have the lowest amount of sea buckthorn oil. As in the case of lipid content, we can conclude that the cause of this discrepancy is the uneven distribution of sea buckthorn oil in the raw material.

The water content shows, as for the other chemical parameters, a significant difference ($p < 0.0001$) between batches. The value of this parameter increases concomitantly with the increase in the content of sea buckthorn oil in the batches.

The pH values for the three batches are extremely close, which can be seen by the insignificant p-value ($p = 0.184$).

CONCLUSIONS

The addition of sea buckthorn oil to meat products and their transformation into functional products is a suitable option to improve their bioactivity and antioxidant capacity, thus responding to market demand for products with improved value and without synthetic additions (synthetic antioxidants). Sensory analysis conducted in this study demonstrated very good consumer acceptability of the sea buckthorn oil injected tenderloin, especially for the 3% and 1% sea buckthorn oil amounts. Injecting the tenderloin with 5% sea buckthorn oil was considered by some evaluators to be too high, but these were in extremely small numbers. Colorimetric analysis showed that increasing the amount of sea buckthorn oil resulted in decreased brightness of the samples and increased red and yellow color. This aspect does not negatively influence the analyzed products, as the presence of a reddish color is expected from meat products, which increases the attractiveness of the products. Chemical analysis revealed a rather high heterogeneity between batches in terms of the macronutrient composition. Significant differences from the other batches were found in the batch with 5% added sea buckthorn oil, with a decrease in moisture content due to a beneficial increase in the lipid profile with the addition of sea buckthorn oil. For the lot with 3% added sea buckthorn oil, lower chemical values were recorded for fat content and higher for moisture content compared to the lot injected with 1% sea buckthorn oil. This is most likely due to the uneven distribution of the sea buckthorn oil in the tenderloin mass, and injection with manual injection devices is not a suitable option for this process.

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ANALYSIS OF THE STATUS OF FOOD AND DRINKS PROTECTED BY GEOGRAPHICAL INDICATION SCHEMES IN ROMANIA AND EUROPEAN UNION

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Abstract

Food production is experiencing nowadays the transition to "smart" products which are processed by new protective techniques or minimally processed; there is also an increasing interest in traditional foods, which are perceived as healthier, and more environmentally friendly. In this regard, EU agricultural policies are based on specific measures for the entire food chain, aimed at reducing food waste and increasing sustainability; this has an essential role in protecting the availability, accessibility, and quality of agri-food products. In this paper, we focused on presenting the main categories of foods and drinks protected by geographical indication schemes PDO (Protected Designation of Origin), PGI (Protected Geographical Indication), TSG (Traditional Speciality Guaranteed), GI (Geographical Indication) at the EU and Romanian levels. We presented the statistics based on eAmbrosia EU and the Romanian Ministry of Agriculture database, the procedure for registration, and the role of this certification for the producers, community reputation, and branding.

Key words*: eAmbrosia database, GI, PDO, PGI, quality schemes, registration, statistics, TSG.*

INTRODUCTION

Consumers appreciate food quality when deciding to buy food which is also important for manufacturers when negotiating food products' prices. Food schemes were implemented and used by the European Commission (EU) in order to protect food products with specific quality characteristics. The EU agricultural policy is focused on the "quality switch" in the food chain which means the orientation of food production towards standardized quality designed foods and on local, traditional, and organic production.

These regulations have a specific regional or local impact on social, economic, and environmental levels and also may determine an increased consumer interest (Poetschki et al., 2021).

There is an increased perception of the EU food schemes for quality in the context of consumers' high concern on health issues and implications (food additives, sugar, and fat content and genetically modified organisms) but still low interest in eco-labeling and EU food certifications (Costea et al., 2012).

EU has developed food quality systems to secure and advocate foods with special characteristics connected to their geographic origin, organic processing, and also for traditional foods. These systems are a useful tool to help highlight the quality and traditions associated with registered products and to reassure consumers that they are genuine products, not imitations that seek to benefit from the good name and reputation of the original (Sadilek et al., 2016; Tregear et al., 2016).

All these quality schemes are labelled with a specific EU logo for origin and quality. The logo indicates that the labelled product has certain characteristics resulting from the geographic location in which it was manufactured or from traditional ingredients or manufacturing processes. The logo also informs customers of the quality, origin, and/or authenticity of the food (Xu et al., 2022).

In addition, most activities related to the production and processing of food products protected by quality schemes take place in rural areas; this can have an immediate benefit. From a cultural perspective, programs that focus on the geographical origins and traditional aspects of food contribute to the preservation and promotion of cultural heritage (including the preservation of gastronomic traditions). As a product becomes more popular with consumers, it can attract tourists who may be interested in the history of the product and the region, how it was made, and the quality control process. Therefore, other relevant sectors (tourism and hospitality) in rural areas can also benefit from these quality systems. Economic development in these areas can also bring other benefits, such as improved infrastructure and public facilities, better use of land and efficient management of natural resources (Dogan et al., 2012).

The PDO and PGI quality schemes grant the exclusive right to use registered and protected product names to the manufacturer who made the initial application and to any other manufacturer who can meet the requirements of the scheme. With regard to PDO and PGI, producers outside the geographic location are expressly excluded from the use of registered names. Using an approved logo allows manufacturers to make informed choices by communicating to consumers the quality and authenticity of their products. Finally, several studies have shown that these programs make an invaluable contribution to sustainable rural development, for example by conserving local crop varieties, supporting rural diversity and social cohesion, and creating employment opportunities (Blakeney et al., 2021; Conneely et al., 2015; Crescenzi et al., 2022).

Food products that are protected due to their geographic origin fall under the EU intellectual property system. This means that the names of these products are protected both in the EU and abroad. Specific protection agreements are often concluded between the EU and third countries to ensure that products are not misused or counterfeited. Conversely, products originating abroad can also be protected within the EU if an agreement is reached (Curzi et al., 2021; Cardoso et al., 2022).

Quality schemes for products and geographic indication items address a chance for the improvement of the small, neighborhood economy, particularly in provincial regions and hindered regions (Muça et al., 2022).

MATERIALS AND METHODS

For this analysis, we have investigated all the types and categories of food and drinks subjected to registration under quality schemes at the EU and national levels. The data was collected from the European Union official website, eAmbrosia database (Figure 1), accessed on 30 March 2023, and from the Romanian Ministry of Agriculture and Rural Development website (https://www.madr.ro/, accessed on 30 March 2023) and CPAC (https://cpac.afir.info/, accessed on 30 March 2023), only for the Romanian products.

Figure 1. Screenshot for the eAmbrosia website, the EU quality systems registry

In the Register of Agricultural and Food Products, foods are classified into different classes: cheese; other products of animal origin (eggs, honey, dairy products except for butter, etc.); fresh meat, meat products (boiled, salted, smoked, etc.); fresh or processed fruits, vegetables and grains; bread, pastries, cakes, confectionery, biscuits, and other bakery products; oils and fats; fresh fish, molluscs and crustaceans and derived products; beer; chocolate and derived products etc. Here is also a Wine register and a Register of spirits. All the quality schemes registered in Romania can be found, in addition to the eAmbrosia database, in CPAC database by the Ministry of Agriculture and Rural Development.

In the CPAC database, in addition to the DOP, IGP, GI, STG, Mountain Product, and Organic Product quality schemes, we can also find other nationally certified quality systems, namely Traditional Product, Established Recipe, and Certified Wines (Figure 2).

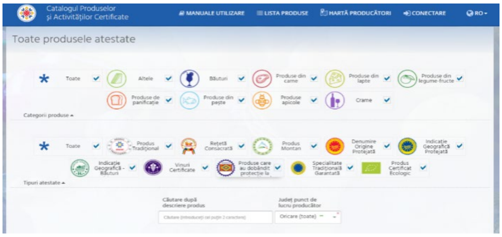

Figure 2. Screenshot for CPAC website, catalog of certified products and activities

RESULTS AND DISCUSSIONS

The food market is now abundant in all types of products and consumers must identify criteria for choosing; these are often price and quality, but EU quality schemes become slowly a new tool to recognize the food quality products, based on sensory characteristics and geographical area (Sperdea, 2015).

The results of this study are centered on the main quality schemes: PDO (Protected Designation of Origin); PGI (Protected Geographical Indication); TSG (Traditional Speciality Guaranteed); GI (Geographical Indication). Based on data extracted from eAmbrozia, we have analysed the situation of each category of products, by status, country, and scheme types.

In the eAmbrosia database (accessed on March 2023), a number of 1942 products from the PDO quality scheme category, 1335 PGI, 61 STG and 257 GI were registered, applied and submitted at the European Union level.

Of the 3595 products with a special indication, 3344 were registered as follows:

- PDO: 1821 products with special indication;
- PGI: 1222 products with special indication;
- TSG: 56 products with special indication;
- GI: 245 products with special indication (Figure 3).

Compared to March 2021, the number of these types of products increased by 10.22% (for PDO), by 16.39% (for PGI), by 10.91% (for TSG) and 2.9% (for GI) (eAmbrozia, accessed May, 2021).

84 PDO quality scheme products, 189 PGI, 6 TSG, and 21 GI were registered, applied, and submitted in non-EU countries IG.

From the total products registered in non-EU countries, only 48 products were PDO registered, 162 registered PGI, 18 registered GI, and 4 products registered TSG (Figure 4).

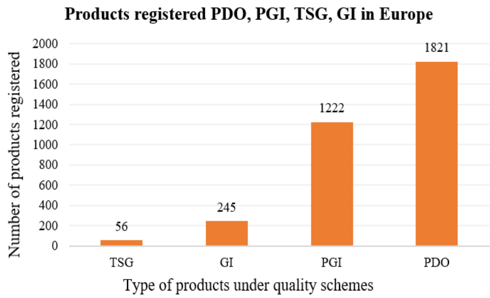

Figure 3. PDO, PGI, STG, and GI products number registered in the European Union

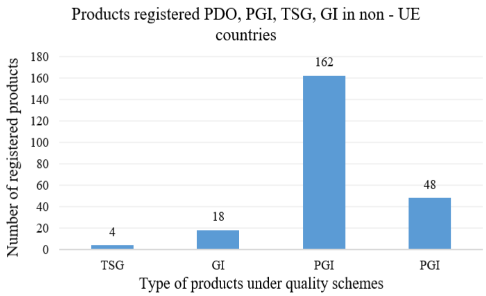

Figure 4. PDO, PGI, STG, and GI products number registered in non-EU countries

PDO (Protected Designation of Origin) products

Products: Food, agricultural products, and wines.

Specifications: All operations from production, processing, and handling should occur in the specific region. This means that the milk for the cheese-making must exclusively come from the region where the cheese is made.

Example: PDO Kalamata olives are produced entirely in the Kalamata region of Greece, using varieties of olives from that area.

Label: Mandatory for food and agricultural products, optional for wines.

Product names registered as PDO are those with the strongest links to the location where they were registered.

In the eAmbrosia database, there were 2.026 registered, published, and applied PDO products in EU with 1.821 registered.

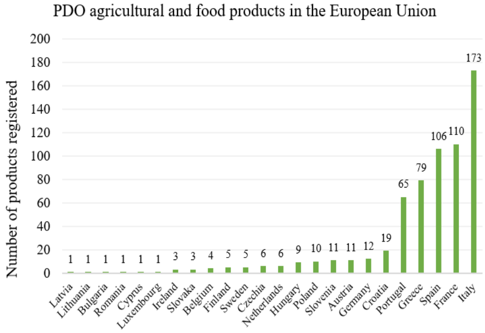

Figure 5. The country-by-country situation of PDO agricultural and food products registered in the EU

Italy with 173 products is the country with the most PDO-registered agricultural and food products (Figure 5) followed by France with 110 and Spain with 106 products, respectively. Romania, has only one registered product in the PDO category, Telemea de Ibănești.

The PDO quality schemes category includes superior wines that present specific characteristics and must meet a series of quality-related requirements: the delimitation of the creation region, the viticultural assortment utilized, the winemaking techniques, the alcoholic strength by least normal volume, the examination and enthusiasm for the organoleptic qualities.

In the category of PDO wines, Italy and France lead the rankings (with 408 wines and 363, respectively).

Romania has 40 wines registered under PDO quality schemes, including Pietroasa, Însurăței, Adamclisi, Cotnari, Murfatlar, Odobești, Sâmburești, Bohotin, Dealu Bujorului, Nicorești, Drăgășani, Cotești (Figure 6).

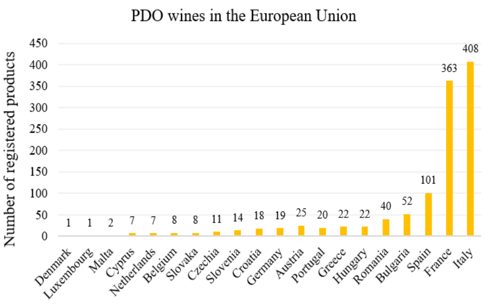

Figure 6. PDO wines registered in the EU countries

Famous French wines can be also found in this category, like vin Sante del Chianti, Pinerolese, Beaumes de Venise, La Grande Rue, Casa del Blanco, Granada, Coteaux Bourguignons, Nero Di Troia.

PGI (Protected Geographical Indication) products

Products: Food, agricultural products and wines.

Specifications: For most items, no less than one of the phases of processing or handling should occur in a separate area. For example, Gouda Holland PGI is made with milk from dairy farms all over the Netherlands, while Noord-Hollandse Gouda PDO is only made with milk from the province of Noord-Holland.

Example: Queso Castellano is a cheese that it is made in Castilla y León made only with raw or pasteurized sheep's milk, 100% from Castilla y León.

Label: Mandatory for food and agricultural products, optional for wines.

PGI places an emphasis on the connection between the name of a certain quality, reputation, or another characteristic that can essentially be attributed to the geographical origin of the products (Glogovețan et al., 2022).

Regarding the situation of agricultural and food products PGI registered, published, and applied, at the EU level, there were 870 products, with 783 registered.

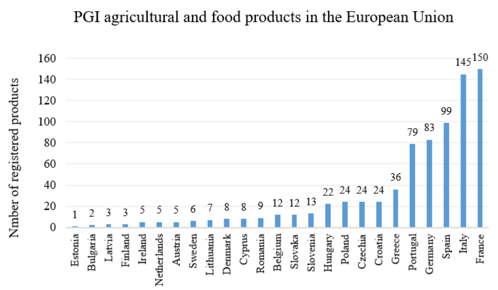

Figure 7. PGI agricultural and food products registered in the EU, by country

France and Italy are top of representatives in this product category: France with 150 PGI-registered agricultural and food products and Italy with 145 PGI-registered agricultural and food products (Figure 7). Romania registered a PGI product on 30.03.2023, Plăcinta Dobrogeană.
At the EU level, there were 439 wines registered as a PGI quality scheme.
As expected, Italy takes first place in the category of wines from PGI quality schemes with 118 wines, followed by Greece with 114 wines.
France also occupies an important place in the ranking of PGI wines, 3rd place, with 75 wines.

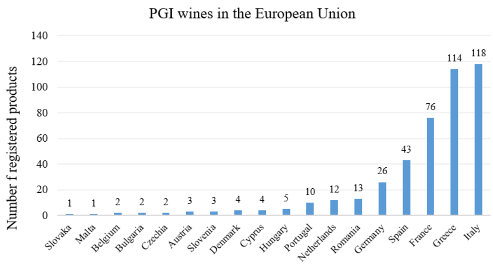

Figure 8. PGI wines registered in the EU, by country

GI (Geographical Indication) products

Products: Spirits and flavored wines.
Specifications: At least one of the distillation or processing steps must take place in that region for the majority of products. However, the raw materials need not necessarily be from the same area.
Example: Nocino is a liqueur of ancient tradition, typical of the province of Modena since 1870. The raw material from which it is obtained, and which characterizes it sensorially, is the walnut hulls, collected before the hardening of the shell, with which an infusion of alcohol is made.
Label: Optional for all products.
If a particular quality, reputation, or another character can essentially be attributed to the geographical origin of the product, then the Geographical Indication (GI) protects the name of a spirit drink or aromatized wine that originates in a country, region, or locality.
The proportion of the production process that must take place in that region or the quantity of raw material that originates from that region is what distinguishes PDO from PGI.
The GI protection is applied only to alcoholic beverages and aromatized wines (Chifor et al., 2022).
At the level of the European Union, in March 2023, there were a number of 245 spirits registered in the eAmbrosia database as a GI quality scheme.
As shown in Figure 9, France occupies the most important place in this category, with 53 varieties of spirits, followed by Germany with 36 and Italy with 35 varieties each.
Following the evolution of processing technologies and developments in food science, consumers tend to be oriented toward traditional foods and recipes, manifesting their national identity, interest in food safety and security, or origin of products. In a study conducted by Petrescu-Mag (2017), it was observed that 67.6% of consumers buy products by the manufacturer's name, 45% take into consideration the country of origin when purchasing foods and 47.5% consider that special labels on the products are important.
Geographical indications and quality schemes can be used by stakeholders and authorities as a tool for quality and product marketing to consumers. It is observed that at EU level and also Romania there is a high concentration of wines with registered geographical specifications (Bichescu et al., 2017).
In the eAmbrosia database, at the EU level number of 56 STG agricultural and food products were registered in March 2023.

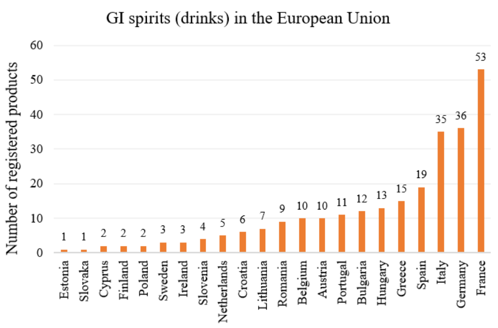

Figure 9. The country-by-country situation of GI-type spirits registered in the European Union

TSG (Traditional specialty guaranteed) products

Products: Food and agricultural products.
Specifications: The minimum period of existence of a product on the market to acquire recognition is 30 years.
Example: Mozzarella Tradizionale TSG is a stringy, soft cheese made from whole fresh cow's milk, produced in southern Italy; its name comes from the verb mozzare, which means "to cut".
Label: Mandatory for all products.

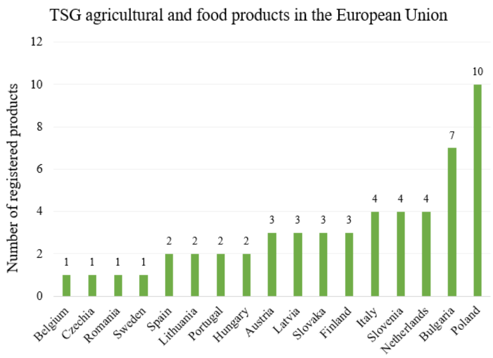

Figure 10. TSG agricultural and food products in the EU countries

TGS products underline the traditional characteristics like the specifics of the processing technology, food composition, its structure, without binds it to a particular geographic region. When a product is registered as an TSG, it is protected from misuse and counterfeiting.
In addition, each member state can use its own set of national and regional quality labels. The logos of the respective national quality schemes can be found on the packaging of products that are protected by those schemes, whether they are in countries outside of the EU or at the EU level (Mariani et al., 2021; Menapace & Moschini, 2021).
The situation of traditional STG products at the European level is shown in Figure 10, where their number for each country is shown.

Thus, Poland and Bulgaria are the countries with the most STG products registered, respectively 10 and 7 products. The Netherlands, Slovenia, and Italy also have 4 products each (Figure 10).
In this product category, Romania has only one registered product, namely traditional salad with carp roe.
Nowadays, the interest of Romanian producers in certification through EU schemes has been increasing and products that were known only at the regional level are becoming recognized in many areas of the country. In order to increase food sustainability, local authorities have implemented procedures for the attestation of traditional food products (Zugravu et al., 2021).
Until March 2023, Romania had registered, published and applied 83 products with special indication, of which 73 were registered, 10 having applied or published documentation; 47 products with a special Romanian PDO indication were submitted, registered, and published (Table 1).

Table 1. Registered and published Romanian PDO products

No	Product name and type	Registration date
1-34	Recaş; Segarcea; Miniş; Dealu Mare; Mehedinţi; Oltina; Aiud; Lechinţa; Drăgăşani; Alba Iulia; Sâmbureşti; Sebeş-Apold; Banat; Cotnari; Iaşi; Iana; Bohotin; Crişana; Nicoreşti; Pietroasa; Dealul Bujorului; Dealul Mare; Panciu; Banu Mărăcine; Târnave; Murfatlar; Huşi; Sarica Niculiţel; Coteşti; Odobeşti; Ştefăneşti; Babadag; Dealu Mare	10/05/2007
35	Adamclisi	30/07/2007
36	Însurăţei	14/11/2018
37	Telemea de Ibăneşti	15/03/2016

Table 2. Applications for PDO Romanian products

No	Product name and type	Application date
1	Jidvei	05/07/2022
2	Drăgăşani	16/08/2021
3	Cotnari	16/02/2023
4	Bohotin	20/07/2021
5	Murfatlar	24/10/2022
6	Iaşi	06/03/2023

25 traditional Romanian PGI products were submitted, registered and published until March 2023 (Table 3).

Table 3. Registered and published Romanian PGI products

No.	Product name	Registration date
1-13	Viile Carașului; Dealurile Munteniei; Dealurile Olteniei; Terasele Dunării; Colinele Dobrogei; Viile Timișului; Dealurile Zarandului; Dealurile Transilvaniei; Dealurile Sătmarului Dealurile Crișanei; Dealurile Munteniei; Dealurile Moldovei, Dealurile Vrancei	28/04/2007
14.	Pleșcoi Sausages (Cârnați de Pleșcoi)	04/10/2019
15.	Danube Smocked Makerel (Scrumbie de Dunăre afumată)	03/12/2018
16.	Săveni cheese (Cașcaval de Săveni)	22/04/2021
17.	Bloated fish from Bârsa County (Novac afumat din Țara Bârsei)	06/04/2017
18.	Topoloveni Plum Jam (Magiun de prune Topoloveni)	08/04/2011
19.	Sibiu Salami (Salam de Sibiu)	19/02/2016
20.	Tulcea pike roe salad (Salată cu icre de știucă de Tulcea)	04/06/2021
21.	Sibiu salted cheese (Telemea de Sibiu)	16/10/2019
22.	Dobrogean pie (Plăcintă dobrogeană)	30/03/2023

Table 4. Applications for Romanian PGI products

No.	Product name	Product type
1.	Terasele Dunării	Wine
2.	Salinate de Turda	Foods
3.	Pecica bread (Pită de Pecica)	

Only 2 traditional Romanian TSG products were submitted, registered, and published until March 2023 as can be seen in Table 6.

Table 5. Romanian GI products registered, published and applied in March 2023

No.	Product name	Registration date
1.	Vinars Vaslui	21/06/2005
2.	Vinars Segarcea	21/06/2005
3.	Horincă de Cămârzana	21/06/2005
4.	Vinars Vrancea	21/06/2005
5.	Pălincă	13/02/2008
6.	Vinars Murfatlar	21/06/2005
7.	Vinars Târnave	21/06/2005
8.	Țuică Zetea de Medieșu Aurit	21/06/2005
9.	Țuică de Argeș	21/06/2005

Table 6. Romanian TSG products registered, published and applied in March 2023

No.	Product name	Status	Registration date
1.	Traditional salad with carp roe	Registered	29/09/2021
2.	Marinated sardine	Applied	

Romania had 73 products registered as PDO, PGI, GI and TSG quality schemes, as follows:

- DOP: 41 foods and wines;
- PGI: 22 foods and wines;
- GI: 9 spirits;
- TSG: 1 food (Figure 11).

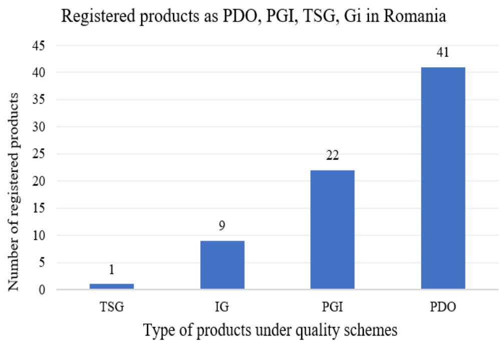

Figure 11. PDO, PGI, TSG, GI products number registered in Romania

CONCLUSIONS

- The interest in the registration of quality schemes protected by certification labels is increasing, at the EU and also Romanian levels.
- More and more producers are following the consumer's interest and need for better, safer, less processed, traditional food products.
- Authorities and stakeholders are getting more prepared and active in supporting producers in their way for getting certifications, as a way of brand protecting, rural and communities developing. The quality schemes of food and drinks certified at EU or national level assure the proper safety of products, but, the risk of contamination in different phases of processing is not completely excluded. In this case, there is very important for the authorities to have tools for conducting controls on the food chain to ensure consumers' safety and security.

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THE EFFECT OF RED LENTIL FLOUR ON THE QUALITY CHARACTERISTICS OF BEEF BURGERS OBTAINED FROM TWO DIFFERENT ANATOMICAL REGIONS

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Abstract

The study was carried out to evaluate the quality of four beef burgers from two different anatomical regions (round and hind shank) that were manufactured in the USV Iasi Meat Processing Workshop. The technological process of obtaining the four types of burgers had as a specificity the addition of red lentil flour in two proportions (5 and 10%) and the adjustment of the proportions of added fat according to the level of added lentil flour (35 and 15% fat). The obtained products were evaluated physicochemically for color, chemical composition, cooking, and sensory parameters to determine the perception of the attributes appearance, aroma, juiciness, tenderness, aftertaste, and off-flavor. Samples with higher percentages of red lentil flour showed lower lightness, lower heat treatment losses, and less diameter reduction. The same samples demonstrated better water retention capacity after cooking, though the type of raw materials used also had an impact on this parameter. According to the sensory evaluation, the addition of lentil flour in combination with the fat content resulted in improved textural attributes (juiciness and tenderness) and the samples showed high acceptability.

***Key words**: beef burgers, beef round, beef hind shank, quality parameters red lentil flour.*

INTRODUCTION

Meat and meat products are generally recognized as essential foods in the human diet due to their nutritional properties, which include proteins with high biological value, essential fatty acids, fat-soluble vitamins, minerals, and bioactive compounds (Yilmaz Önal et al., 2021). However, despite these elements that give it its nutritional potential, meat is deficient in carbohydrates, especially non-starch polysaccharides such as fiber (Câmara et al., 2020; Mehta et al., 2015). Furthermore, the fat (especially saturated fat) and cholesterol content of meat contribute to its unfavorable reputation (Salejda et al., 2022). Nowadays, consumer concern for health has increased, with choices being oriented towards meat products with beneficial health properties (Kambarova et al., 2021). For these reasons, more and more research has been done to study ways to improve meat products by adding plant-based materials, thus obtaining functional products. Functional foods are those foods that, in addition to nutritional intake, benefit the body's biological functions, improve overall health, and reduce the risk of certain diseases (Illippangama et al., 2022; Kausar et al., 2019).

Fibre intake in the body is achieved through the consumption of cereal products, legumes, fruits and vegetables in sufficient quantities to reach the recommended dietary fiber intake of 25-30 g per day (Amine et al., 2003; Mann, 2007; Miller, 2021; Vasyukova & Lyubimova, 2022). However, most people do not reach the recommended dietary fiber intake, since due to rapid urbanisation and dietary style changes, people consume more processed, fast food, high cholesterol and high-calorie snacks (Zaini et al., 2020).

Hence, the meat industry is constantly looking for solutions to meet consumer demands related to health, quality of life and sustainability, with the addition of plant fiber in meat products being some of the ways to improve quality both from a nutritional point of view and shelf life (through the antioxidant properties of the fiber sources used) (Fernández-López et al., 2021; Pame et al., 2022; Salejda et al., 2022). Moreover, the introduction of a plant fiber source in meat products leads to improved technological properties through improved cooking yield, reduced fat and salt content, and improved texture and structural properties (Kambarova et al., 2021; Kausar et al., 2019; Kim & Paik, 2012).

Lentil (*Lens culinaris*) is a high-protein legume (20.6 - 31.4%) and is an excellent source of essential amino acids (except methionine and cystine), fiber (11% in green and 31% in red), minerals and bioactive compounds (Bayomy & Alamri, 2022; Hajas et al., 2022; Oduro-Yeboah et al., 2022). The use of lentil flour in various food products such as bakery products, dairy products, and meat products has attracted the attention of producers and consumers due to its balanced nutritional composition and functional properties (solubility, gelation, emulsification, foaming) (Argel et al., 2020; Romano et al., 2021).

Burgers are minced meat products with high consumer acceptability and are frequently consumed. In the formulation of these products, binding agents and elements that increase water-holding capacity are currently being used to improve the cooking yield and juiciness of the product (Shariati-Ievari et al., 2016).

The study carried out followed the effect of adding lentil flour in proportions of 5 and 10% in the manufacture of four experimental batches of beef burgers derived from two different anatomical regions (beef round and shank), correlated with the proportion of fat added (15% and 35%), on the sensory and physicochemical characteristics of the batches studied.

MATERIALS AND METHODS

The study was carried out in the Meat Processing Workshop and the Meat and Meat Products Technology Laboratory of the Faculty of Agriculture of the University of Life Sciences in Iasi.

The raw materials used to manufacture the two experimental batches of burgers were purchased from the local food market, as was the red lentil flour used in the formulation of the products. The raw and auxiliary materials required in the technological process to produce 1 kg of product for the four assortments are presented in Table 1. The experimental protocol consisted of two categories of raw materials: beef round and hind shank, two levels of fat (15 and 35%) and red lentil flour (5 and 10%). The other ingredients were added in the same proportions in all samples: salt (2.2%), onion (1.5%), black pepper (1%), mustard (0.5%), chilli (0.2%) and sweet paprika (0.5%).

The raw meat was passed through a grinder through a 0.8 mm diameter sieve. After obtaining the meat paste, burgers of equal size (12 cm diameter and 2.5 cm height), weighing ~200 g were formed, then packed in trays and wrapped in polyethene film. The products were stored under refrigerated conditions (2-4°C) until the proposed analyses were performed.

Table 1. Formulations to prepare the raw beef burgers that included red lentil flour

Batch code	**Ingredients (%)**				
	Beef round	**Beef hind shank**	**Pork backfat**	**Red lentil flour**	**Total**
L1P5	60	-	35	5	100
L2P10	75	-	15	10	100
L3R5	-	60	35	5	100
L4R10	-	75	15	10	100

Chemical characterisation was carried out by determining the proximate chemical composition. Moisture, fat content, and protein were determined using a spectrophotometer (FoodCheck analyzer) using an infrared light source. The physical characterisation of the burger samples consisted of instrumental color determination, evaluation of cooking yield, losses and diameter reduction after heat treatment. The samples' color was determined with the portable Konica Minolta Chroma Meter CR-410, in the three-dimensional CIE color system, measuring the L*, a* and b* color parameters with the D65 illuminant at an observation angle of 10 degrees. The instrument was calibrated on a white calibration plate for standard values before starting the measurements. The cooking yield was calculated according to formula (1). The difference between the sample's weight before and after cooking, as calculated using formula (2), served as the basis for calculating cooking losses. Diameter reduction was determined according to the method presented by do Prado et al. (2019), using formula (3).

$$\text{Cooking yield (\%)} = \frac{\text{weight of cooked sample}}{\text{weight of raw sample}} \times 100 \quad (1)$$

$$\text{Cooking loss (\%)} = \frac{\text{weight of raw sample} - \text{weight of cooked sample}}{\text{weight of raw sample}} \times 100 \quad (2)$$

$$\text{Diameter reduction (\%)} = \frac{\text{raw sample diameter} - \text{cooked sample diameter}}{\text{raw sample diameter}} \times 100 \quad (3)$$

The sensory evaluation of the burger samples was carried out on a group of 58 evaluators aged 21-25, students of the University of Life Sciences of Iasi, in the Sensory Analysis Laboratory of USV Iasi. Six tasting sessions were organized, at the beginning of each session the evaluators were trained on the tasting procedure and on how to complete the sensory evaluation questionnaire. The burgers were previously cooked on a grill for 15 minutes until they reached a temperature of 90°C in the thermal center. The cooked pieces were transversely sectioned into eight portions, which were coded with a three-digit code and distributed to the evaluators for analysis. For each product formulation, evaluators were asked to score from 1 to 10 the following sensory descriptors: appearance, aroma, juiciness, tenderness, after taste and off-flavor.

RESULTS AND DISCUSSIONS

The results obtained for the color parameters (L*, a*, b*) of the burger samples, as well as the p-values of the variation factors and their interactions, are shown in Table 2. The lightness L*, which represents the reflecting diffusivity, measured from 0 (black) to 100 (white), recorded the highest value for sample L3R5 (46.98 ± 1.37). The addition of red lentil flour in the higher proportion resulted in lower lightness in the burger samples ($p < 0.01$). Moreover, the percentage of fat also showed a significant effect ($p < 0.01$) on the lightness of the burger batches. Similar results have also been reported by adding lentil flour to meatballs or coating beef burgers with lentil powder (Serdaroglu et al., 2005; Embaby et al., 2016).

Hence, the lower L* lightness of L2P10 and L4R10 samples can be explained by the addition of a higher percentage of red lentil flour, which is correlated with the lower percentage of fat added in the production process.

Regarding the a* values (redness), a significant influence of the anatomical region of the raw material ($p < 0.001$) as well as the amount of fat in the sample ($p < 0.01$) on this parameter was observed. In this context, samples L1P5 and L2P10, made from beef round, showed a higher intensity of red color compared to samples L3R5 and L4R10.

Table 2. Colour parameters for raw burgers

Burger samples	L*± SD	a*± SD	b*± SD
L1P5	46.50 ± 2.01	6.67 ± 0.32	14.52 ± 1.09
L2P10	44.01 ± 1.09	7.31 ± 0.13	14.37 ± 0.44
L3R5	46.98 ± 1.37	5.48 ± 0.44	14.88 ± 0.77
L4R10	44.43 ± 1.35	5.83 ± 0.08	16.22 ± 0.12
p-value			
A (Meat anatomical region)	0.510 ^{ns}	**<0.0001** ***	**0.003** **
B (Fat, %)	**0.002** **	**0.001** **	0.076 ^{ns}
C (Red lentil powder, %)	**0.001** **	0.098 ^{ns}	0.193 ^{ns}
AB (Meat anat. reg. x Fat, %)	0.965 ^{ns}	0.247 ^{ns}	**0.031** *
AC (Meat anat. reg. x Red lentil powder, %)	0.610 ^{ns}	**0.009** **	0.569 ^{ns}
BC (Fat, % x Red lentil powder, %)	**0.001** **	0.160 ^{ns}	0.184 ^{ns}

ANOVA Tukey test: ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

In contrast, the color parameter b* (yellowness) showed higher values in samples L3R5 and L4R10 (14. 88 ± 0.77 and 16.22 ± 0.12, respectively), with the meat anatomical region factor and the interaction meat anatomical region x fat (%) determining distinctly significant ($p<0.001$) and significant ($p<0.05$) differences between experimental burger samples.

The mean values obtained for the chemical composition (± SD) of uncooked and cooked burger samples are shown in Table 3.

The protein content before cooking was significantly different ($p<0.05$) between the four formulations, with the samples with the higher amount of lentil flour and lower fat proportion showing higher amounts of protein. Protein levels showed an increase after heat treatment, with a significant ($p<0.05$) increasing trend only in the L2P10 sample made from beef round with 10% lentil flour. Similar results were obtained by Baugreet et al. (2016) and Serdaroglu et al. (2005) by introducing legume flour into beef burgers and meatballs.

The lipid content identified in the samples analyzed before cooking ranged between 14.06 ± 0.208 (L4R10) and 28.53 ± 0.603 (L1P5), showing significant differences ($p<0.05$) between samples. After cooking, the burger samples showed a fat content ranging from 13.73% ± 0.252 (L2P10) to 26.73% ± 0.153 (L1P5). There was a non-significant increase ($p>0.05$) in fat content for batches made from the beef hind shank, while batches made from beef round lost significant amounts of fat during heat treatment ($p<0.05$).

The moisture content of burger samples was negatively correlated with lipid content, thus samples with 35% added fat had lower amounts of water in the composition compared to samples with 15% added fat. Moreover, following heat treatment, a significant ($p<0.05$) increase in moisture was observed in the samples from beef round, while the experimental batches from hind shank showed statistically insignificant water loss ($p>0.05$).

Table 3. Chemical composition of burgers before and after cooking

Burger samples	**Before cooking**				**After cooking**			
	Fat (%)	**Moisture % (%)**	**Protein (%)**	**Collagen (%)**	**Fat (%)**	**Moisture % (%)**	**Protein (%)**	**Collagen (%)**
L1P5	28.53 ± $0.603^{d,y}$	54.63 ± $0.513^{a,x}$	15.76 ± $0.058^{a,x}$	13.66 ± $0.321^{a,x}$	26.73 ± $0.153^{cd,x}$	55.80 ± $0.100^{a,y}$	16.30 ± $0.000^{a,x}$	14.33 ± $0.058^{a,x}$
L2P10	19.86 ± $0.321^{b,y}$	61.43 ± $0.208^{c,x}$	17.83 ± $0.153^{b,x}$	15.93 ± $0.208^{c,x}$	13.73 ± $0.252^{a,x}$	66.10 ± $0.200^{cd,y}$	19.23 ± $0.058^{b,y}$	17.46 ± $0.115^{b,y}$
L3R5	25.73 ± $0.404^{c,x}$	56.73 ± $0.252^{b,x}$	16.43 ± $0.058^{ab,x}$	14.50 ± $0.200^{ab,x}$	25.93 ± $0.666^{c,x}$	56.50 ± $0.520^{ab,x}$	16.50 ± $0.173^{a,x}$	14.53 ± $0.208^{a,x}$
L4R10	14.06 ± $0.208^{a,x}$	65.83 ± $0.208^{d,x}$	19.10 ± $0.100^{c,x}$	17.30 ± $0.436^{d,x}$	14.50 ± $0.265^{ab,x}$	65.46 ± $0.208^{c,x}$	19.10 ± $0.000^{b,x}$	17.26 ± $0.058^{b,x}$

a, b, c, d - The same superscript letter within the same column means there is no significant difference between any two means ($p>0.05$). x, y, - The same superscript letter within the same row means there is no significant difference between the same parameter analysed before and after cooking ($p>0.05$).

The properties of the burger samples after cooking are shown in Table 4.

The whole cooking losses ranged from 7.00% (L2P10) to 13.40% (L3R5), and the cooking yield varied inversely with the losses, ranging from 86.60% (L3R5) to 93.00% (L2P10).

Samples L2P10 and L4R10 achieved the highest cooking yields, being the formulations with the lowest fat percentage and the highest red lentil flour.

The lower cooking yields of L1P5 and L3R5 can be attributed to excessive fat separation.

Table 4. Cooking parameters of burger samples

Cooking parameters	L1P5	L2P10	L3R5	L4R10
W_r (g)	0.182	0.200	0.194	0.198
W_c (g)	0.160	0.186	0.168	0.180
D_r (g)	11.5	11.5	11.5	11.5
D_c (g)	9.5	10	9.5	10.8
Cooking loss (%)	12.09	7.00	13.40	9.09
Cooking yield (%)	87.91	93.00	86.60	90.91
Diameter reduction (%)	17.39	13.04	17.39	6.09

W_r - weight of raw sample; W_c - weight of cooked sample; D_r - raw sample diameter; D_c - cooked sample diameter.

A positive correlation can be observed between cooking yield and protein content in the samples. Taking into account the ability of protein to retain fat as well as the fiber intake contained in red lentil flour (with oil binding and oil retaining capacity), the addition of 10% lentil flour resulted in lower cooking losses.

During heat treatment, the products tend to shrink due to the loss of water and fat as well as the denaturation of the contained proteins. The reduction in diameter after cooking was lowest in sample L4R10 (6.09%), while samples L1P5 and L3R5 showed equal values in terms of reduction in diameter (17.39%).

The quality of a food product also includes the consumer acceptance element. In this regard, the burger samples were sensory evaluated on a 10-point scale for attributes such as appearance, aroma, juiciness, tenderness, after taste and off-flavor (Table 5). Based on the individual scores, a mean score was calculated, and an ANOVA test was applied to determine the degree of influence of the variation factors (A, B, C) on the perception of the panel of evaluators. A significant influence of added fat and lentil flour content and the interaction between these factors on the perception of the evaluators was observed.

Texture attributes like juiciness and tenderness improved with the increasing percentage of added lentil flour, while after-taste and off-flavour attributes scored higher for these samples due to the evaluators' perception of a more intense red lentil flavor.

Table 5. Sensory analyses of burger samples

Sensory attributes	L1P5	L2P10	L3R5	L4R10	Significance levels of p-value					
					A	B	C	AB	AC	BC
Appearance	7.16±1.224	8.22±0.850	6.72±1.164	8.31±0.900	ns	***	***	ns	***	***
Aroma	8.76±1.171	8.53±0.991	8.76±1.264	8.27±1.250	ns	*	*	ns	ns	*
Juiciness	8.09±0.996	8.91±0.874	7.82±1.114	8.76±1.246	ns	***	***	ns	***	***
Tenderness	7.56±0.693	8.47±1.236	7.22±1.042	8.07±1.095	ns	***	***	*	***	***
After taste	5.09±1.125	5.73±0.986	5.27±1.250	5.87±1.014	ns	***	***	ns	***	***
Off flavor	5.11±1.481	5.53±0.991	5.38±1.614	5.67±0.977	ns	ns	ns	ns	ns	ns

A = Meat anatomical region; B = Fat, %; C = Red lentil powder, %; ns = not significant ($p > 0.05$); * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

CONCLUSIONS

The addition of red lentil flour had an impact on the physicochemical characteristics of beef burger samples. Increasing the percentage of red lentil flour decreased the lightness L* of the burger samples while at the same time increasing the intensity of the red color (a* value). The percentage of lentil flour introduced, associated with the amount of fat in the samples, increased the moisture and protein content. Cooking losses were higher for formulations with lower levels of red lentil flour, with the most favorable cooking yield obtained for sample L2P10, made from beef round. The sensory evaluation revealed significant differences in the variation factors of the experimental batches, but the samples showed high acceptability from the evaluators.

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TECHNOLOGICAL INDICATORS OF GOAT'S MILK AS A RAW MATERIAL FOR CHEESE PRODUCTION - SURVEY

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Abstract

Cheese is a product with a high nutritional value, compared to milk, because of its low water content, presence of lactic acid and cooking salt. The yield, composition and organoleptic qualities of cheese are influenced by production technology and milk composition, which varies depending on the breed, the season, the lactation stage, the type and proportion of fodder in the goat ration, and other factors. The quality of raw milk, along with the cheese preparation technology, have an impact on some physicochemical parameters and the proteolytic processes during cheese ripening. The casein fraction of the milk protein is a dominant factor affecting curd density, the syneresis rate, the moisture content and ultimately the cheese quality and yield. The technological characteristics of goat's milk coagulum differ from those of cow's milk, as the differences are mostly related to the genetic polymorphism of α_{s1}-casein. The purpose of this survey is to analyze the technological indicators of goat's milk and some factors related to the quality of the finished cheese product.

Key words: *cheese, goat’s milk, technological indicators.*

INTRODUCTION

Cheese is one of the ancient forms of manufactured food production, dating back to 5-6000 BC. Depending on the geographical location, the milk composition and the technologies used, a large assortment of goat cheese is produced in the world, ensuring high profitability for the producers. Nowadays, cheese is an essential food product and an integral part of a healthy diet, and its low levels of lactose make it suitable for people with digestive disorders. This shows its significance as a functional food, whose physiologically active components provide both nutritional and health benefits (Hasler, 2000).
In many countries, goat's milk is most often processed into cheese, alone or in a mixture with cow and sheep’s milk (Iliev & Michailova, 2014; Poveda et al., 2008; Janštová et al., 2010; Miloradovic et al., 2017). Over 400 types of goat cheese have been described with over 700 names of chese, which are produced by cow, sheep and buffalo’s milk in combination with goat’s milk (Park et al., 2017). France, Spain and Greece are the largest producers of goat’s milk in Europe (respectively 24.6%, 17.5% and 16.0%) and cheese from this raw material (France - 47.1%, Greece - 21, 3%, Spain - 20.3% (Medina and Nuñez, 2017). The quality of raw milk is determined by its chemical composition and sanitary-hygienic characteristics (Sharma et al., 2011; Petzer et al., 2017).
The criteria for milk quality and the threshold values of the indicators evolve depending on the development of science, production technologies and consumer preferences.
Casein genotype is the factor of greatest significance for the overall variation in curdling parameters (Lawrence, 1991; Albenzio et al., 2009).
Data from a number of studies show that goat’s milk high in αs1-casein has higher dry matter content, milk fat, protein, casein, phosphorus, lower pH and better curdling properties than that containing less αs1-casein (Ambrosoli et al., 1988; Barbieri et al., 1995).
The potential of milk to influence cheese yield is highly dependent on composition, particularly fat and protein content (Brito et al.,

2002; Guo et al., 2004).Cheese composition changes depending on the stage of lactation and corresponds to changes in milk composition (Soryal et al., 2005). The season of milk collection has an effect on the variation of chemical and coagulation characteristics of milk (Zullo et al., 2005), as well as farming systems, temperature treatment of milk, even the time of milking during the day (Zullo et al., 2005; Galina et al., 2007.

Cheese quality is related to the ripening process in terms of proteolysis, lipolysis and glycolysis. The purpose of ripening is to transform the fresh cheese into different types of cheese with specific organoleptic characteristics defining its type and quality. The composition and biochemical characteristics of raw milk, technological operations, the curdling process, the preparation of the curd and the ripening of the cheese directly or indirectly affect the quality of dairy products (Albenzio & Santillo, 2011), corresponding to the intensity of the ripening process from the point of view of lipolysis, proteolysis and glycolysis.

Hygiene control during milk production is also of great significance for the optimization of the hygienic and nutritional characteristics of goat cheese and the proteolysis occurring during the ripening process of the product.

RESULTS AND DISCUSSIONS

In the present survey, the technological indicators of goat's milk and some factors affecting the quality of the final cheese product are observed.

Casein genotype

The technological characteristics of goat's milk rennet coagulum differ from those of cow's milk. The technological disadvantages of goat's milk arise from its low casein content, in particular α_{s1}-casein, and its high βcasein content. Differences in goat's milk casein content are associated with the α_{s1}-casein genetic polymorphism. Milk containing genetic variants A, B and C is distinguished by the highest amount of α_{s1}-casein, E - medium, D and F low, and 0 - does not contain it (Grosclaude et al., 1994). Goat' milk that is low in αs1-casein gives a less dense rennet coagulum (Ambrosoli et al., 1988), which is associated with greater whey casein losses and lower cheese yields (Pirisi et al., 1994).

These characteristics of the rennet coagulum in goat's milk limit the variety of cheese types from this milk, most of which fall into one of the following groups (Medina & Nuñez, 2017):

- Fresh (white) unrepented cheese with a low dry matter content, usually below 25%;
- Soft cheese produced mainly from lactic acid coagulum, small in size, cylindrical or pyramidal in shape, usually with mold growth on the surface;
- Semi-hard or hard cheese produced with a predominance of rennet coagulum, larger in size, with a flat cylindrical shape and a dry rind on the surface.

In dairy goats, casein fractions (αs1- and αs2-caseins) vary both between breeds and within individuals of the same breed and can affect cheese yield and rheological properties of the gel (Pirisi et al., 1994; Delacroix-Buchet et al., 1996).

Goat's milk from animals with strong alleles is associated with higher cheese yield and firmer curd than milk from animals with weak alleles (Albenzio et al., 2009; Clark and Sherbon, 2000; Tziboula-Clarke, 2003).

Total protein and αs1-casein content correlate with coagulation time (Clark & Sherbon, 2000; Mastewet et al 2012), which is highly dependent on milk pH values. Milk with a low as1-casein content has a higher pH and a significantly lower total protein content (Johansson et al., 2015).

In milk with a low concentration of as1 casein, the coagulation time ss 15% longer and the curd is 60% lower in density, compared to milk with a high content of as1 casein (Johansson 2015). Comparing the cheese yield from milk with and without as1 casein content, Pierre et al. (1998) found that the yield of milk containing casein was 26% higher than that of milk without casein. In addition, the organoleptic test on the 2nd and 13th day of ripening shows that the specific "goat smell" is significantly weaker in the cheese produced from milk containing as1 casein. A similar dependence between cheese yield and casein content in milk was found by Soryal et al. (2005), who indicated that the higher casein content in the milk of Nubian goats increased cheese yield by 60% compared to that of the milk of Alpine goats.

Effect of temperature treatment of milk

Miloradovic et al. (2017) studied the impact of temperature treatment of goat's milk at 80°C and 90°C with a delay of 5 min, instead of the traditional - 65°C/30 min on the composition, quality and yield of white brined cheese (0.005% - w/v *Lactococcus lactis* subsp. *lactis* and *L. lactis subsp. cremoris*). The higher temperature treatment of goat's milk significantly affects the composition and yield of cheese. It leads to binding of more water, fat and whey proteins in the curd mass and increasing the yield. For the studied period - 24 hours, 10, 20, 30 and 40 days of production, the content of dry matter in the goat's white brined cheese produced by the traditional technology (65°C/30 min) decreased from 44.27 to 40.34, 38.76, 39.82 and 38.16%.

The milk fat content and milk fat in dry matter of this batch of cheese changed from 18.50 to 17.67, 18.00, 18.83 and 17.50% and 41.14 to 43.75, 47.55, 45.89 and 45.86%, respectively, in contrast to the water content of the non-fat residue, which it gradually decreases during the ripening period of Feta goat cheese. Miloradovic et al. (2017) found an increase in this indicator for white brined cheese from the 24th hour to the 40th day of production - from 67.52 to 72.46% (10th day), 72.22% (20th day) 73.32% (30th day) and 74.92% (40th day). As a result of the high-temperature treatment of goat's milk, the water in the fat-free residue of the goat's cheese is higher already at the 24th hour of production (74.41% - 80°C/5 min and 75.81% - 90°C/5 min) and almost does not change until the 40th day (73.89 and 74.80%, respectively).

The content of salt and salt in the water phase of the fresh cheese produced by the traditional method have higher values already at the 24th hour (1.24 and 2.24%, respectively) and gradually increase to 3.28 and 5.27% at the 40th day of production. The salt content of the experimental batches of fresh white brined cheese was lower (1.09% at 80°C/5 min and 90°C/5 min), reaching values of 3.09 and 3.39% on the 40th day of production. The salt content of the aqueous phase in the fresh cheese of the experimental batches was lower (1.76% at 80°C/5 min and 90°C/5 min), gradually increased to levels close to the control cheese during the 10th÷30th day and reaches 5.19 and 5.67% at the end of ripening (40th day). The authors found that a higher pasteurization temperature (80°C/5 min and 90°C/5 min) inhibited the degradation of α_s-casein but did not significantly affect that of β-casein fraction.

Barac et al. (2016) also investigated the physicochemical composition of white brined cheese produced from goat's milk at high temperature processing at 90°C/10 min. The cheese at 24th hours of production was 46.71% dry matter, 57.18% milk fat in dry matter, 36.58% protein in dry matter and pH 6.28. During ripening, the pH decreases to 4.6 on the 30th day, which practically does not change on the 40th and 50th days with pH 4.57. During the first 10 days of ripening, initial intensive degradation of αs-casein is established - over 50.70% till the 10th day and extremely slowly during the remaining 40 days. A slow and prolonged degradation of β-casein was observed, the residual amount of which (85.14%) was significantly higher than that of α_s-казеина (47.85%).

Impact of lactation phase and season on milk collection

Cheese produced from milk obtained at the end of lactation had a higher fat, protein and dry matter content, which corresponds to the higher values of these parameters in milk during that lactation phase (Soryal et al., 2005).

Higher cheese amount was registered from the milk of Alpine goats in October compared to July and September. The amount of cheese from Nubian goats increased as milk lactation progressed (Soryal et al., 2005). The total fatty acid content was high in the summer months (July, August, September). During the early lactation stages (May and June) and the last stage of lactation (October), the total concentration of fatty acids in the cheese was about half of that in August. This may be explained by the heat of the summer months, when there is an increase in fat consumption with the ration and a high level of lipolysis (Palmquist et al., 1993), leading to an increase in the relative proportion of long-chain fatty acids in milk. At the same time, the short-chain fatty acids (C4:0, C6:0 and C8:0) in the cheese did not show significant variation during lactation, which corresponded to a minor variation in organoleptic qualities.

López et al. (2012) also noted the season effect on cheese composition and fatty acid profile, especially during the summer months, despite the lack of reliable differences between individual proteolysis parameters. Cheese produced in spring had a better physicochemical composition, lower NaCl content and a better fatty acid profile. The pH values were lowest in autumn (5.44) compared to spring and summer (5.65). Dry matter was highest in cheese produced in summer (61.75 %) and lowest in cheese produced in spring (59.44 %). Season has no effect on the content of most fatty acids except C12:0, C14:0, C16:0, C18:0 and C18:1

The farming system corresponds to the composition of the milk and cheese because of the different composition of the ration. The cheese obtained from the milk of pasture-raised goats is characterized by a higher content of linoleic acid (163 mg/100 g vs. 142 mg/100 g cheese) and tocopherol (211 mg/100 g vs. 87 mg/100 g cheese) and lower fat content (12.3 mg/100 g vs. 16.9 mg/100 g cheese) and cholesterol (63.2 mg/100 g vs. 80.4 mg/100 g cheese), compared to cheese from goats raised in a barn (Galina et al., 2007).These results confirm that the components of nutritional and health interest are contained in significantly higher concentrations in cheese produced from pasture-raised animals compared to barn-raised ones. Cheese obtained from pasture-raised animals is characterized by a higher content of unsaturated fatty acids, antioxidants and aromatic compounds and a lower cholesterol content, compared to that from the milk of animals raised in a barn (Rubino & Chilliard, 2003; Chilliard et al., 2005; Luna et al., 2005; Nudda et al., 2005; Cabiddu et al., 2006).

The increase of concentrated feed in the ration of goats from 10 to 40 % is accompanied by a reliable increase in milk productivity. The content of long-chain and polyunsaturated fatty acids, as well as conjugated linoleic acid and omega-3 fatty acids was significantly higher and the ratio of omega-6:omega-3 fatty acids was lower in cheese obtained from the milk of goats that received less concentrated feed (Volkmann et al., 2014).

Even the time of milking has an effect on the milk's cheese making potential. Milk obtained from morning milking is distinguished by better coagulation qualities, higher coagulation speed and better curd consistency (Zullo et al., 2005). The raising system of goats and the hygiene of the dairy also have an impact on the microflora and, accordingly, on the composition of goat’s milk and cheese. (Albenzio et al., 2006).

Syneresis is one of the critical processes of great significance for cheese quality (Dejmek & Walstra, 2004), which can be defined as the separation of whey from the curd, stirring and heating. Coagulation and syneresis conditions determine the final characteristics of the cheese because of their impact on moisture and protein content. Dry matter content, whey composition and final product characteristics are determined to a significant extent by the control of the syneresis process and mechanical and physical whey separation during cheese processing (Garcia et al., 2014).

Milk coagulation, induced by yeast, is influenced by the type and concentration of culture and enzyme, incubation temperature and milk composition (Raynal & Remeuf, 2000). Curd density is the most significant indicator that determines the yield and quality of cheese and, accordingly, the economic efficiency of production (Clark & Sherbon, 2000). Better coagulant density reflects the formation of a denser network, which is related to the amount and composition of casein (Dimassi et al. 2005). Dense curd improves cheese yield by stimulating the retention of milk constituents (Martín-Hernández & Juarez, 1992).

Cheese yield, defined as the amount of cheese produced from a given amount of milk (Zeng et al., 2007), is one of the main factors determining the efficiency and profitability of cheese making (Emmons, 1993).

Cheese production corresponds to many genetic, physiological and production-technological factors such as milk characteristics, protein and fat content, genetic variants of proteins, somatic cells, conditions of cheese production, milk treatment, homogenization of fat, type of coagulation, the use of different starters, curd density, tub design, curd treatment (Fenelon & Guinee, 1999; Mona & Nawal, 2011).

Proteolysis and lipolysis are of greatest significance in the development of the organoleptic qualities of cheese and are controlled by the residual enzyme in the curd, milk proteinase and lipase, proteolytic and lipolytic enzymes from starter and non-starter bacteria, and lipases associated with certain coagulants (Collins et al., 2003; Visser, 1993). Enzymes naturally present in milk (plasmin system, cathepsin D, elastase and lipase) determine the biochemical processes during cheese ripening, and proteolytic and lipolytic enzymes are of greatest significance for cheese making.

Proteolysis is the most complex and significant biochemical process in cheese ripening that determines the texture, aroma and flavor of the products (Fox, 1996). Proteolysis in cheese can be divided into primary and secondary proteolysis. The main process in the first phase is the degradation of casein to polypeptides (McSweeney & Sousa, 2000; McSweeney et al., 2017). The following proteolytic processes (secondary proteolysis) lead to the formation of low-molecular polypeptides and free amino acids that determine the aroma and taste of the cheese.

The rennet from goat kids, often produced by the traditional method on the farms itself, contains chymosin as the main curdling agent and is the main source of lipolytic enzymes such as lipases and esterase (Bustamante et al., 2000) and the applied methods of its preparation can have an impact on the character and intensity of lipolysis. This yeast contains lipolytic enzymes including pregastric and gastric esterases responsible for the release of short- and medium-chain free fatty acids in the cheese (Jacob et al., 2010). The excessive content of these acids imparts a rancid, hot and unpleasant taste to the cheese, which repels consumers (Soryal et al., 2005).

The fat content of brined goat's milk cheese increased from 50.67 at the beginning to 54.87 g/100 g at 60th day, whereas the total protein in the ripening process decreased from 45.2 g/100 g at the beginning to 36.9 g /100 g at the end of the period, as the decrease was most significant during the first 10 days (Miroljub et al., 2013). The degree of proteolysis increased from 3.47% at the beginning of ripening to 11.61% on the 60th day. The water-soluble protein increased from 9.77 to 17.88 g/100 g of cheese on the 60th day of ripening. Both casein fractions (αs-1 and β-casein) are exposed to proteolysis but to different extents. In the ripening process, αs1 casein disappears, while on the 60th day of ripening, the contents of αs2 and β casein decrease by 38.90% and 30.72%, respectively. As the ripening takes place in a salt brine, the decrease in total protein is probably also related to the diffusion of loosely bound proteins and/or partly of partially hydrolysed proteins in the cheese. Analogous changes in the cheese composition were also observed by Mas et al. (2002), noting a continuous increase in dry matter, with values at 3, 15, 30 and 60 days of ripening being 51.96%, 55.28%, 56.19% and 58.90%. The NaCl content gradually increased from 1.46% on the 3rd day to 2.50% on the 60th day of ripening. The fat content also increased from 51.74% at the beginning to 52.64% on the 60th day of ripening. Proteolysis products also increase with cheese ripening. The pH values decreased sharply after the 30th day of ripening and increased gradually until the 60th day. Most of the lactose in the milk passes into the whey, and the remaining lactose is rapidly metabolized in the early ripening stage by the starter and non-starter microflora of the cheese (Jeleva, 2005).

Mallatou et al. (2004) investigated the proteolytic processes in Teleme cheese from sheep, goat and a 1:1 mixture of both. The cheese was produced after standardization of sheep's milk to 6.1, goat's milk to 4.4 and the mixed one to 4.7% fat content. The casein/fat ratio (C/F) in processed milks is in the range of 0.72÷0.75, 0.62÷0.64 and 0.68÷0.69, respectively. A significantly lower degradation of αs-casein was found in goat’s milk cheese than in sheep’s or a 1:1 mixture of them at all stages of ripening at 20th, 60th, 180th and 360th days of production. No significant differences were found in free fatty acids among the three types of cheese.

Water-soluble components, such as mineral substances, water-soluble vitamins, amino acids, lactose, etc., are dissolved in water and all microbiological, chemical and enzyme-chemical processes take place in it during the production, ripening and storage of different types of cheese, therefore the water content is decisive factor for product consistency (Kojev, 2000b).

Proteolysis in brined cheese in the ripening process is significantly influenced by the temperature, related to the general development of lactic acid bacteria and biochemical processes in cheese. In buffalo cheese ripening at a temperature of 9°C, the proteolytic processes are significantly slowed down. The ripening regime at 12°C allows a moderate proteolytic process, with the cheese reaching the indicators for the desired degree of maturity by the 60th day. The high temperature regime of 15°C contributes to the acceleration of proteolytic processes and reaching a desired level of maturity already on the 45th day of ripening (Balabanova et al., 2014).

Hygiene control during milk production affects proteolytic processes. Processing of milk with a high somatic cell count is associated with an increase in the level of proteolysis and a change in the proteolytic pattern in cheese (Coulon et al., 2004). An increased number of somatic cells can impair the cheese making qualities of milk by causing a prolongation of coagulation time, a decrease in coagulum density, an increase in moisture content and, accordingly, a decrease in cheese yield and fat content (Albenzio et al., 2006). The flavor is determined by its taste and aroma, which are the result of the appropriate balance and concentration of numerous aromatic components perceived during cheese consumption. Excessive proteolysis and lipolysis can deteriorate cheese quality and to cause unpleasant nuance due to the high content of bitter peptides and volatile free fatty acids that have an impact directly or as precursors other ingredients (Broadbent et al., 2002; Pinho et al., 2004).

CONCLUSIONS

The technological indicators of goat's milk directly affect the quality and organoleptic properties of the finished cheese product. Rennet coagulum is influenced by casein genotype. The composition of raw milk corresponds to various factors and, along with the production technology, has an effect on the cheese yield. Establishing the relationship between the technological processes and the composition of the milk makes it possible to optimize the production and to achieve better economic results, and from there for a higher profitability of the producers.

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RESEARCH ON THE PHYSICO-CHEMICAL AND MICROBIOLOGICAL QUALITY OF FAST FOOD PRODUCTS

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Abstract

This study aimed to monitor the quality of food products distributed in fast food restaurants. Physico-chemical and microbiological analyses were conducted on burger and sandwich products containing beef, chicken, turkey, fish, cheese, as well as on sweet desserts such as apple juice, ice cream, and milkshakes. Additionally, the equipment and utensils used in the preparation of these products were checked microbiologically. The ice and drinking water from the supply network, used in the preparation process of these products, were tested both microbiologically and physic-chemically.

Key words: *burger, dessert, meat, physico-chemical parameters.*

INTRODUCTION

Human nutrition represents one of the fundamental pillars of its construction. Health and individual balance are directly correlated with food. Adopting an adequate diet, along with other correct behavioral elements, leads to a healthy life. The human body remains healthier when the diet is more balanced. A diversified and individualized diet (based on lifestyle, gender, activity level, health status), correctly composed in terms of calories and respecting the optimal ratio of main substances (carbohydrates, proteins, lipids, dietary fibers), can be considered a balanced diet. In supplying the population with high biological value food products, products of animal origin play a particularly important role, with meat and meat derivatives being a priority. In the context of the observed increase in meat products production, special attention must be given to the quality of the end products. Quality control of the end products should be carried out as quickly as possible to avoid any possible non-conformities (Banu et al., 1980; 1985).

In this context, the present study aimed to monitor the quality of fast food products from a physico-chemical and microbiological perspective. It is well known that not all food is beneficial to the human body. Today, phrases like "unhealthy food" or "healthy food" have almost entered everyday speech. The quality of food depends on both its source and the way it is processed, whether it is done industrially or at home (Ciocârlie et al., 2002).

The foods available on the market today can be categorized as natural, semi-natural, or even semi-synthetic. Among all these, it is well known that the healthiest foods are those of natural origin, which do not contain synthetic additives, have not undergone industrial refining processes, and have not been excessively processed from a culinary perspective (Banu et al., 2009).

Unprocessed natural foods, typically of plant origin and known as raw foods, have particular importance for human health. Food is not just a source of matter and energy; it also carries information (Marcu et al., 2008).

Humans, like any other beings on Earth, are part of the food chains, which means that through their food, they exchange information, matter, and energy with the environment. The emerging field of nutrigenomics has provided evidence of how food influences the genetic information encoded in our DNA and RNA chains. Detached from nature and immersed in a synthetic civilization, modern humans risk severely distorting not only their immediate health but also the fundamental material of their chromosomes through highly processed food products, with consequences that are still incalculable for both themselves and their descendants. Thus, the act of nourishment becomes a matter of great responsibility for the future of the entire human species, as we already live in a world where children are sicker than their parents and, in far too many cases, die before them. Foods, depending on their origin, industrial processing technology, and culinary preparation methods, have distinct compositions, both in terms of the quantities of bioactive compounds and their ratios. In addition to their chemical composition, culinary preparations are distinguished by the ingredients used.

The criteria for classifying foods are extremely varied. From the multitude of classifications, I have chosen a few more conclusive ones so that anyone can get an idea about food. Food is edible substances that contain a certain number of organic elements such as proteins, lipids, carbohydrates, as well as minerals and vitamins. They also consist of water and indigestible substances such as dietary fibers.

Based on their origin, foods can be broadly categorized into plant-based and animal-based. Furthermore, both plant-based and animal-based foods can be divided into multiple groups. Together or separately, these foods have been categorized over time in various forms, upon which numerous diets have been constructed (Mencinicopschi & Cironeanu, 2006; Ciocârlie et al., 2002).

There are also classifications that focus on the value of other nutrients such as carbohydrates and lipids, both in terms of quantity and assimilation power. Additionally, classifications have been developed based on the energy value (caloric content) of food. Although there are sophisticated categorizations in this regard, in general, we can divide food based on its consistency. Thus, we have light foods (snacks) and substantial foods (energy-rich meals).

MATERIALS AND METHODS

Approximately one-third of the ingredients used in Fast Food units in Romania come from local suppliers, such as buns and bread slices, pork ham, salads, jams, apple juices, eggs, and milk. The rest of the ingredients are imported, such as beef, potatoes, chicken and turkey meat, fish, and cheese.

The sampling should be carried out by qualified personnel in accordance with the applicable regulations, from slaughterhouses, meat processing units, distribution networks, and public and collective administration units. Sterile instruments should be used, and each sample should be placed in a sterile container, parchment paper, or new plastic bags. Quantitatively, the proportions specified in the relevant legislation should be respected.

Depending on the product being analyzed, the sampling of the samples was conducted as follows:

- for boneless meat products (e.g., smoked beef, beef and pork pastrami, catering products), thicker portions or slices were collected, including both the surface layers and the deeper layers;
- for not portioned minced meat, samples were collected from each container;
- for meat and meat products packaged as individual units with a certain weight or meat pieces not exceeding 2 kg, 300 g was collected;
- for packaged meat products in membranes and meat products in pieces, whole packaging or pieces were collected;
- for salted or smoked meat products, pieces from the vicinity of the bone were collected.

Packaging of the samples

Carcasses or pieces exceeding 2 kg are packaged in plastic bags, which are sealed and labeled.

Samples from pieces weighing less than 2 kg, if they are in properly sealed packaging, do not require additional packaging; otherwise, they must be packaged in a sterile and tightly closed manner to ensure the possibility of sealing and labelling.

The samples were appropriately labelled, specifying the name and address of the producing unit, the product name, the place and date of sampling, the lot number, and the number of the sampling protocol. The protocol was signed by the person who collected the sample and a representative of the interested party or parties.

Transportation was ensured in a timely manner and under optimal temperature conditions.

Frozen samples were stored in the refrigerator at a maximum temperature of 5°C for a maximum of 12 hours for thawing.
The microbiological analysis of fast-food products under study involved the determination of microorganisms such as *Listeria monocytogenes*, *Salmonella*, and *Enterobacteriaceae*. For water, ice, and the equipment used in the preparation of fast-food products, determinations were made for E. coli, Total Mesophilic Aerobic Bacteria (TMAB), Enterobacteriaceae, and Coliform Bacteria. (Diaconescu & Şuler, 2017; Ionescu & Diaconescu, 2010)
Chemical analysis for tap water samples from the network. The determination of total residual chlorine is based on the iodometric method, which involves the oxidation of potassium iodide by chlorine and the titration of the elemental iodine formed with sodium thiosulfate. Total hardness can be determined by several methods, with the most commonly used one being the complexometric method. The principle of the method lies in the ability of calcium and magnesium ions to form stable complexes with complexon III (disodium salt of ethylenediaminetetraacetic acid) at pH 10. The identification of nitrites and nitrates is based on the use of the GRIESS reagent (a solution of sulfanilic acid and naphthylamine in acetic acid) (Hodosan et al., 2007).

Colony count method or pour plate method

Prepare serial dilutions: 10 g of each product was cut it into small pieces and mixed well with 90 ml of peptone water, to ensure proper dilution. This process is repeated for multiple dilutions, ranging from 10-1 to 10-6, using a new pipette for each dilution and transferring the appropriate amounts to obtain the next dilution. 2 ml from each dilution were distributed into Petri dishes (2 plates per dilution), using the same pipettes used for homogenizing the dilutions. 14-15 ml of molten glucose and yeast extract agar cooled to 45-50ºC were poured over the inoculated plates and mixed the inoculum well with the medium by gently swirling the plates, then allow them to solidify on the benchtop.
The plates were inverted and incubated at a temperature of 30˚C for 48 hours.
Counting and expressing the results was done using a magnifying glass.
TMAB was calculate by multiplying the colony count by the dilution factor and expressing it as colony-forming units per milliliter (CFU/ml) or colony-forming units per gram (CFU/g) if the sample was initially diluted in a known volume of diluent.
The presence and number of coliform bacteria, including the *Escherichia coli* (*E. coli*) species, can be determined using a method called the Most Probable Number (MPN) test.
1 ml of the homogenized product is inoculated and each dilution in test tubes containing one of the enrichment media (Kessler);
Multiple tubes are prepared with different dilutions of the sample, ranging from 10-1 to 10-5. The inoculated tubes are incubated at 37°C for 24 hours. The selective medium encourages the growth of coliform bacteria and suppresses the growth of other bacteria. The appearance of gases in the Durham tube is monitored daily;
If gas is produced, indicating the presence of coliform bacteria, a confirmation test is performed to identify the presence of E. coli. This involve subculturing the positive tubes onto a differential medium such as Levine agar poured into Petri plates and incubation at 37ºC for 24 hours; Colonies grown on Levine agar are checked. E. coli shows dark colored colonies, with a surface with a metallic luster and a golden-green reflex. The other coliform bacteria show dark blue colonies without metallic luster or atypical, opaque, mucous, pink colonies (*Klebsiella*), with a gray-brown center. Colonies specific to coliform bacteria must be differentiated from Salmonella colonies, which are transparent;
For confirmation from two or more characteristic colonies of *E. coli*, a culture is sown in three test tubes with the following media:
- a test tube with BBLV (or lauryl sulfate) and fermentation tube;
- a test tube with tryptonized water heated to 45ºC;
- a test tube with inclined nutrient agar;

The test tubes with the seeded culture media will be incubated at 45ºC for 24 hours. The temperature of 45ºC acts selectively on *E. coli*, favoring the germ in competition with the associated microflora;

After incubation, the cultures are examined for the presence of gases appearing in the Durham tube in the test tube with BBLV medium (Brilliant Green Bile Lactose) and for the presence of indole in the test tube with tryptonized water seeded and incubated by adding a few drops of Kovacs reagent (red ring on the surface of the medium) . If cultures are positive, it is considered *E. coli* confirmation;

From the culture present in the slanted agar, this bacterium is further tested for enteropathogenicity by the rapid agglutination reaction on the slide with polyvalent anti-*E. coli* serum and the sero-group is established using monovalent anti-"O-B" serum.

The genus *Salmonella* belongs to the family Enterobacteriaceae and the isolation and identification of bacteria from the genus Salmonella is done in accordance with SR ISO 65-79/1997.

The pre-enrichment step involves inoculating the samples into a non-selective liquid medium (buffered peptone water). 25 g of the product is inoculated into 225 ml of pre-enrichment medium, and it is incubated at 35 or 37°C for 16-20 hours. This method is applied for frozen or dehydrated products.

The enrichment step is carried out by transferring the sample to two selective enrichment media: Rapaport-Vassiliadis medium, incubated at 42°C for 24 hours, and selenite cystine broth, incubated at 35-37°C for 24-48 hours.

The isolation step involves inoculating two selective media with the cultures obtained from the enrichment step. The following media were used: phenol red agar and brilliant green-Edel Kampelmacher agar.

Incubation is carried out at 35-37°C for 20-24-48 hours. Only the characteristic colonies developed on the selective isolation media are taken into consideration.

In the confirmation stage, presumptive positive colonies of *Salmonella* were tested for biochemical and serological characteristics. Incubation was carried out at 35-37°C on multipurpose media, including TSI (triple sugar iron agar), MIU (motility, indole, urea), and MILF (motility, indole, lysine decarboxylase, phenylalanine deaminize).

After incubation, the inoculated agar medium is examined.

After the biochemical tests, serological confirmations are performed.

RESULTS AND DISCUSSIONS

Microbiological analysis of the 5 types of Fast Food products examined involved the identification of bacteria from the genus *Listeria monocytogenes, Salmonella,* and *Enterobacteriaceae* (Table 1).

Table 1. The results of the microbiological analysis conducted on the analyzed fast food products

Microbiological parameter	**Unit**	**Reference analytical method**	**Analised products**					
			beef burger	**chicken burger**	**cheese burger**	**fish burger**	**turkey burger**	**pork sandwich**
Salmonella	CFU/ 25 g	SR ISO 6579-1:2017	Absent	Absent	Absent	Absent	Absent	Absent
Enterobacteriaceae	MPV/g, maxim	SR ISO 21528-1:2017	0	0	0	0	0	0
Listeria monocytogenes	CFU 5 g, maxim	SRN EN ISO 11290-1:2017	Absent	Absent	Absent	Absent	Absent	Absent

CFU - colony-forming unit
MPV - mean platelet volume

The results of the microbiological analyses conducted by the Laboratory of Physicochemical and Microbiological Analyses for the products under study indicate that all the analysed varieties met the sanitary and veterinary requirements for food safety. The results of the microbiological analyses conducted on some equipment and utensils used in the preparation of FAST FOOD products, specifically the Total Mesophilic Aerobic Bacteria (TMAB) count and Coliform Bacteria, are presented in Table 2.

Table 2. Results of microbiological analyses on equipment and utensils used in fast food preparation

Microbiological parameter	Unit	Reference analytical method	Analysed equipment and utensils			
			Kitchen dressing table 10/10 cm^2	Serving spoon	Tomato cutter blade 10/10 cm^2	Work apron 10/10 cm^2
Total Mesophilic Aerobic Bacteria	CFU/cm^2 CFU/ml	SR EN ISO 4833-1:2014	< 1/cm^2	< 1/ml	2/cm^2	2/cm^2
Coliform Bacteria	CFU/10 cm^2 CFU/ml	SR ISO 4831:2009	Abs/10 cm^2	Abs/ml	Abs/10 cm^2	Abs/10 cm^2

The analysis of these microbiological parameters indicates that the risk of contamination with pathogenic microorganisms in utensils and production equipment is manageable, both through the implementation of sanitation programs and the education of workers in the respective units. The results of the microbiological analyses conducted on water and ice used in the preparation of FAST FOOD products, specifically Escherichia coli, Coliform Bacteria, and Total Germ Count, are presented in the Table 3.

Table 3. The results of the microbiological analyses conducted on drinking water and ice used in the preparation of FAST FOOD products

Microbiological parameter	Unit	Reference analytical method	Prepared ice 1×500 l	Purchased ice 1×500 l	Tap water
Escherichia coli	CFU/100 ml	SR EN ISO 9308-1:2015	0	0	0
Coliform bacteria	CFU/100 ml	SR EN ISO 9308-1:2015	-	-	0
Total Germ Count	CFU/ml	SR EN ISO 4833-1:2014	190	7	-

Drinking water from the network and the ice used in the technological process of obtaining the studied FAST FOOD products meet the sanitary-veterinary requirements for food safety. Physico-chemical analyses were also performed on samples of drinking water from the network. The results of these analyses are presented in Table 4.

Table 4. Physico-chemical parameters determined on samples of tap water from the network

Specification	Reference analytical method	Obtained values	Maximum allowed concentrations
Nitrites (mg/l)	SR ISO 7800-3/2000	0.935	Max. 50
Nitrate (mg/l)	SR EN20777/2002	0.0246	Max. 0.5
Chlorides (mg/l)	SRISO9297/2001	20.10	Max. 2500
Total hardness (d)	SR ISO 6059/2008	6.75	Min. 6.
Turbidity (NTUs)	SR EN ISO 7027/2001	0.44	Max. 5
Conductivity (µS/cm, 20^{0}C)	SR EN 27888/1997	252.6	2500
ph units	SR EN ISO 10523/2012	7.7	6.5-9.5

NTUs - Nephelometric Turbidity Units

The analysis of the values presented in this table highlights that the water samples subjected to the study met all the analyzed parameters, thus the tap water meets all the drinking water requirements. Microbiological analysis was also conducted on some sweet dessert preparations such as apple juice, chocolate ice cream, and chocolate syrup shake. The results are presented in Table 5 and represent the average of five determinations.

Table 5. Results of microbiological analysis performed on sweet dessert preparations.

Microbiological parameter	Unit	Reference analytical method	Dessert type		
			Chocolate ice cream (5×250 g)	Shake with chocolate syrup (5×250 g)	Apple juice
Escherichia coli	CFU/ml	SR EN ISO 9308-1:2015	<10	<10	-
Coliform bacteria	MPV/g	SR ISO 21528-1:2017	<10	<10	0
Total Germ Count	CFU/ml	SR EN ISO 4833-1:2014	6×10	9.4×10^{2}	-

Also in this case, the results of the microbiological analysis performed on the three types of sweet dessert preparations studied indicate that all the analyzed varieties met the sanitary-veterinary requirements for food safety.

CONCLUSIONS

Physicochemical and microbiological analyses were conducted on both fast food products and sweet dessert products. Additionally, the microbiological analysis was performed on the equipment and utensils used in the preparation of these products, as well as on the ice and tap water (both microbiologically and physicochemically) used in the technological process of producing the studied fast food products.

For all analyzed fast food products, the manufacturing recipes align with those specified in the specialized literature for these types of meat preparations. From an ingredient perspective, all the studied products are manufactured according to the recipe and working instructions.

Beef, chicken, turkey, and fish used in the products come from a network of farms where animals are fed non-genetically modified cereals, grains, and grass. These undergo 31 quality control points from the farm to the distribution center, and 3 control points in restaurants to monitor the quality and safety of the raw materials.

The monitored and analyzed microbiological parameters for the studied products meet the required standards, allowing the examined products to be marketed and consumed without restrictions.

Both tap water and ice used in the technological process of producing the studied fast food products meet the sanitary-veterinary requirements for food safety.

From a physicochemical perspective, the tap water used in the respective establishments meets all the drinking water standards.

The analysis of these microbiological parameters indicates that the risk of contamination with pathogenic microorganisms is manageable through the implementation of sanitation programs and the education of workers in the respective units.

Proper preparation of products and adherence to food safety practices are daily concerns food units.

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VEGETABLE DERIVATIVES USED IN MEAT PRODUCTS

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Abstract

Plant derivatives are high biological value products, with sensory properties appreciated by consumers, which are easily obtained, relatively cheap and can be successfully used as meat analogues. Additionally, some plant derivatives result as waste from the vegetable products industry and can be successfully valorized in meat compositions for their content in vitamins, antioxidants, unsaturated fats, minerals or fibers. Consequently, when used in the meat industry, they can improve the structural, sensory and nutritional characteristics of the finished products. Due to their high fiber content, they can be successfully used to increase yields, but also to reduce the potential caloric value of meat products, with positive effects on health. The paper aims to analyze scientific research referring to the use of plant derivatives as meat analogues and their effects.

Key words*: meat products, nutrients, plant derivatives, yield.*

INTRODUCTION

The studies developed in this scientific work started from the hypothesis that plant extracts with a high content of specific phytochemical substances, obtained from various plant materials, will have synergistic antioxidant and antimicrobial effects, will inhibit the growth of pathogenic and spoilage bacteria, the evolution of oxidative processes in various meat products and will improve the quality and safety of meat.
Plant derivatives can be used to improve the technological characteristics of meat, increase yields, improve the rheological properties of meat compositions, and enhance the sensory characteristics of finished products.
Furthermore, it was assumed that incorporating complex plant extracts with antioxidant properties into processed meat products would result in healthier products due to the reduction of oxidation levels in the meat, thus preventing inflammatory reactions without significantly affecting their sensory characteristics.
The main objective of the studies was to improve the functional value of meat products by adding powders and natural extracts with antioxidant properties, as meat is a healthy food product, containing proteins with high biological value, a high content of essential minerals and B-group vitamins. The translation is formal, technical, in paragraphs, detailed, and advanced.
Lipid oxidation represents one of the causes of meat and meat product deterioration as it is accompanied by the appearance of a large number of unwanted changes in flavor, texture, and nutritional value. The speed of lipid oxidation can be effectively reduced by using antioxidants. Synthetic antioxidants have been widely used in the meat industry, but consumer concerns about the safety and toxicity of products have led the food industry to seek natural alternatives.
As a result, some natural ingredients, including herbs and spices, have been studied, especially in Asian countries, as potential antioxidant products in meat and meat products.
Research has shown that natural antioxidants extracted from plants can be used as alternatives to synthetic antioxidants due to their equivalent or even greater effect on inhibiting lipid oxidation. It has been demonstrated that certain compounds from herbs and spices contain many phytochemical substances that have antioxidant, anti-inflammatory, and anticancer activities (Boruzi, 2020).

Currently, consumers are increasingly concerned with all aspects that can contribute to improving the quality of life, and although dietary intake is not the only element that influences well-being and health, it is one of the most important.

The factors that have favored this evolution include the current extremely high impact on public opinion of media reports regarding the relationship between diet and health, the increase in life expectancy of the population, or a heightened attention to disease prevention.

This concept also includes food products known as "functional foods". These are defined as foods that help prevent and treat certain conditions and diseases, in addition to their nutritional value as such.

In fact, this is not a new idea, for centuries humanity has been using the properties of certain foods that provide additional physiological benefits to treat, alleviate or prevent diseases.

RESULTS AND DISCUSSIONS

The influence and effects of natural extract additives on the physico-chemical and sensory characteristics of meat products

Some of the research that has followed the use of plant derivatives in the creation of new meat products is presented in Table 1.

Boruzi (2020) demonstrated that the powder of walnut leaves used as a source of natural antioxidants in pork meatballs had a considerable antioxidant activity.

The study aimed to evaluate the oxidative stability and color stability of ground pork meat containing walnut leaf powder at levels of 0.2% and 0.5% addition, compared to a control without antioxidant addition and a control with 0.1% butylated hydroxytoluene addition.

The results showed that the moisture content was higher in the samples containing walnut leaf powder, indicating that it contributed to the retention of moisture in the product.

The color of the meat depends on the concentration of meat pigments, as well as the physico-chemical properties of the meat substances and those added to it. The addition of walnut leaf powder as a natural antioxidant, with a high content of phenolic compounds, had a significant effect on color stability.

Increasing the storage time resulted in a decrease in the overall acceptability of pork meatballs, with or without additives. However, samples with an addition of 0.5% walnut leaf powder showed higher scores for overall acceptability and flavor compared to the control, as the powder has the potential to reduce oxidative rancidity and extend the shelf life of cooked pork meatballs. Additionally, increasing the amount of walnut leaf powder significantly increased the free radical scavenging activity.

Boruzi (2020) also found that adding cherry stem extract did not significantly affect the brightness and autochthonous microflora activity of the meatballs, but improved the antioxidant activity.

Another natural derivative used for the sensory valorization of pork meat is represented by essential oils.

Mantzourani et al. (2023) studied the use of thyme and oregano essential oils alone or combined with ethanolic extracts of pomegranate in pork meat.

The sensory evaluation of pork loins during storage at 4°C for 7 days, treated with red wine marinade with various combinations of ethanolic extract of pomegranate, as well as essential oils of oregano and thyme, was studied in terms of color, tenderness, flavor, and juiciness.

The study showed that the most intense color was recorded for pork fillets with the addition of pomegranate extract compared to pork fillets marinated with essential oils (oregano and thyme) and red wine, and in terms of tenderness and juiciness, no significant differences were recorded. The sensory impact of marinades on pork fillets was accepted by consumers.

Turgut et al. (2016) found an improvement in the stability of sensory characteristics (color and odor) of beef meatballs in which pomegranate peel extract was added, extending the shelf life by up to 8 days.

Increasing the TVP percentage from 10 to 40% in pork sausages resulted in a decrease in sensory acceptability, with the control sample being the best scored by tasters (Hidayat et al., 2018).

Table 1. Plant derivatives used in meat products

Authors	**Product type**	**Used derivative**	**Processing conditions**	**Antioxidant activity**	**Antimicrobial activity**	**Sensory properties and functional properties**
Boruzi, 2020	Pork meatballs	Extracts from walnut leaves and cherry stems	Treating at 72^0C in the thermal center	The leaves/extracts have increased antioxidant activity comparable to synthetic antioxidants.	Poor	Positive effects; color deterioration is reduced during storage. The flavor has improved. The succulence has increased.
Boruzi, 2020	Pork meat	Powder from walnut leaves, extracts from walnut leaves and cherry stems	Storage, $0\text{-}4^0C$	-	Significant (does not inhibit bacteria, but a weaker colony growth is observed)	-
Mantzourani et al., 2023	Pork fillets	Thyme essential oil (TEO) and oregano essential oil (OEO) alone or combined with pomegranate extracts.	Treating at 72^0C in the thermal center Storage, $0\text{-}4^0C$	-	Fewer pathogenic bacteria	Color, tenderness, flavor and juiciness
Turgut et al., 2016	Beef meatballs	Pomegranate peel extract	Refrigerate, 4 ± 1^0C	Significant antioxidant activity	-	The color and smell of staleness have been improved
Biasi et al., 2023	Product Mortadella type.	Blueberry flour	Refrigerate, 0 ± 4^0C	Increased antioxidant activity	-	-
Baune et al., 2022	Burgers and meatballs	Texturized vegetable protein (corn, soy and pea protein)	Baking at 70^0C	-	-	Improved cooking loss, moisture retention, shrinkage, cohesiveness, chewing, elasticity, hardness and cutting resistance
Bakhsh et al., 2021	Beef meatballs		Baking at 70^0C	-	-	A significant reduction in hardness, cohesion, and thickness, but also an increase in dietary fiber content; total moisture and fat content was lower, and water release and cooking losses were reduced
Hidayat et al., 2018	Hybrid sausages	Textured Pumpkin Seed Protein	Treating at 72^0C in the thermal center	-	-	Increased water content due to high retention capacity of TSPS; sensory quality of products decreased
Revilla et al., 2022	Fresh meat from pork pulp; pork back fat	Chickpea protein isolate	Refrigerate, 0 ± 4^0C	-	-	Significantly improves water-fat binding capacity and improves textural properties (hardness, elastic ability and chewing)
Wang et al., 2019	Beef and pork products imitating vegetable protein	8 common edible mushrooms= analogues of meat protein	Treating at 72^0C in the thermal center	-	-	The texture, taste and flavora of the product have been greatly improved
Stephan et al., 2018	Vegetarian sausages	Mushrooms species *P. sapidus*	Treating at 72^0C in the thermal center	-	-	Texture close to traditional sausages, with better sensory value than other vegetable proteins
Patinho et al., 2021	Beef burgers	Mushrooms species *A. bisporus*	Baking at 70^0C	High oxidative stability	-	Good sensory properties, good fragrances

The influence of plant derivatives on lipids and meat pigments oxidation

Boruzi (2020) studied the global antioxidant activity of samples treated with powder and extracts of walnut and cherry stems, which was significantly higher ($P<0.05$) even compared to samples with added butylated hydroxytoluene and also improved the degree of color deterioration.

After 15 days of storage, the antioxidant activity was significantly higher in samples with added extracts rich in phenolic compounds and walnut leaf powder (with a higher content of flavonoids) compared to control samples with or without butylated hydroxytoluene. The walnut leaf extract showed a higher antioxidant capacity than the cherry stem extract (3.67 mmol/100 g Trolox for walnut leaf extract and 2.84 mmol/100 g Trolox for cherry stem extract). The results showed that an addition of 0.2% walnut leaf powder has a delaying effect on lipid oxidation similar to the addition of 0.1% butylated hydroxytoluene, with positive effects on shelf life.

Turgut et al. (2016) studied the antioxidant effect of pomegranate peel extract on lipid and pigment oxidation in beef meatballs during storage at a temperature of 4 ± 1°C.

The lyophilized extract from pomegranate peel was incorporated into freshly ground beef at concentrations of 0.5% and 1% and compared to a reference control of 0.01% butylated hydroxytoluene and a control with no antioxidant.

It has been demonstrated that in the tests with the addition of pomegranate peel extract, with a high content of phenolic compounds, the value of acid thiobarbituric reactive substances, peroxide formation, loss of sulfhydryl groups, and protein carbonyl formation were lower than the control ($P <0.01$) after 8 days of storage.

An alternative natural derivative with antioxidant effects is blueberry flour, which has been shown to have superior effects on digestibility, antioxidant capacity, and preservation of mortadella-type products over time. Biasi et al. (2023) found the formation of peroxides on the 7th day of storage in control samples (made from pork), while in samples containing blueberry flour (*Vaccinium corymbosum* L. species), peroxide formation began on the 15th day of storage.

The addition of blueberry flour (0.05%), which has a high concentration of phenolic compounds, mainly chlorogenic acid and isoquercetin, controlled the oxidation of mortadella lipids during storage. Antioxidant activity increased in the intestinal phase with the increase in the concentration of added blueberry flour.

The influence and effects of natural extract additives on microbial activity

Analyzing the effect of damage caused by walnut leaf powder, walnut leaf extract, and cherry stem extract on microbial activity, Boruzi (2020) found that they did not have a bactericidal effect on the studied species, but instead a weaker colony development and selection of microbial genera was observed. Thus, in samples with walnut leaf extract, the lowest microbial load was found, consisting mainly of *Lactobacillus* and *Staphylococcus* genera, in samples with cherry stem extract, *Bacillus cereus* developed mainly, while in those with walnut leaf powder, *Brochothrix thermosphacta* and *Bacillus* spp. developed.

Mantzourani et al. (2023) studied the effect of thyme and oregano essential oils and pomegranate extracts on the microbiological stability of pork meat. The concentration of Enterobacteriaceae and mesophilic bacteria decreased compared to the control when adding wine, oregano essential and thyme essential oil, while there were no statistically significant differences in the concentration of yeasts and molds. On the other hand, the evolution of staphylococci was reduced by the added extracts, indicating a strong antimicrobial effect. Pseudomonas bacteria were affected by the wine and oregano essential oils mixture. The added extracts did not have a negative influence on the organoleptic properties.

The influence of natural plant extracts on the functional properties of meat

Baune et al. (2022) have argued that textured vegetable proteins such as corn and soy blend, pea protein used as an alternative in meat products significantly alter the technological properties of meat.

Due to the strongly varied techno-functional properties of vegetable proteins (such as water and oil binding, gelling capacity, and gel resistance), they can be incorporated into meat products, with dry texturized vegetable protein being less efficient than those with high moisture content. Baune et al. (2022) have shown that a mixture of soy protein isolate, wheat gluten, and starch (ratio 5:4:1) is suitable for obtaining 100% plant-based meatballs.

It has been demonstrated that extrusion conditions have had a significant impact on the properties of meatless meatball, such as loss during cooking, moisture retention, cohesiveness, mixing, elasticity, hardness, and cutting resistance (Samard et al., 2021).

The addition of 10-40% textured vegetable proteins resulted in lower values for cohesiveness and hardness, and an increase in gumminess and chewiness values compared to beef meatballs, while water release and cooking losses were reduced (Bakhsh et al., 2021).

Ebert et al. (2021) analyzed the buffering capacity of pork meat compositions (188 mmol H/kg*ΔpH) compared to wet pea (225 mmol H/kg*ΔpH), pumpkin (333 mmol H/kg*ΔpH), and sunflower (259 mmol H/kg*ΔpH) textures, and found a slight increase in buffering capacity for vegetable textures, possibly related to the increased amount of amino acids with high buffering capacity and mineral residue content.

The water holding capacity slightly increased with the partial replacement of meat with 10-40% textured soy protein in pork sausages. At the same time, the hardness of the samples decreased with the increase in the percentage of added texturized protein (Hidayat et al., 2018). This can be attributed to the improved hydrophilic character of the product due to the high water retention capacity of soy and pea proteins (Hidayat et al., 2018).

Revilla et al., 2022 studied the effect of stability of meat emulsions in which meat was replaced with 25% to 100% of chickpea protein isolate and bacon was replaced with olive oil. They found a reduction in cooking losses ($P < 0.05$) and centrifugation losses up to 50% substitution. Increasing the chickpea protein isolate addition to 100% resulted in increased cooking and centrifugation losses.

The addition of chickpea protein isolate (0.5-2%), as a replacement for polyphosphates, in pork meat products resulted in a decrease in cooking loss of 13.29%, 13.33%, 12.57%, and 0.76% compared to the control group. At the same time, the increase in chickpea protein isolate concentration generated a direct proportional increase in hardness and gumminess, and a decrease in springiness and cohesiveness (Wang et al., 2023). Heating promotes the unfolding of protein conformation and exposure of reactive groups of chickpea protein isolate and meat proteins (Wang et al., 2023), which favors their interactions and the formation of a complex gel network that helps to retain structure, reducing water and fat expulsion during cooking.

The nutritional advantages of natural extracts/protein analogues on meat

Wang et al. (2003) studied the nutritional advantages of edible mushrooms and concluded that they can become a major source for obtaining meat analogues.

Mushrooms are an important source of nutrients, with a high protein content ranging from 18.1-53.7%, and rich in unsaturated and polyunsaturated fatty acids at 17.3-66.7% (reported as total fatty acids). They are also an important source of vitamins (173-782 mg sterols/g dry substance) and minerals at 5-12%, reported as dry substance (Kalač, 2009). Mushrooms are a source of antioxidants such as ascorbic acid, beta-carotene, lycopene, and gamma-tocopherol. The total phenolic content for *Pleurotus ostreatus*, recorded by Chirinang et al. (2009), was 42.47 GAEs/g dry substance. Mushroom proteins are complete proteins, containing all essential amino acids necessary for the human body, some of which have therapeutic effects in treating gastritis, esophageal cancer, diabetes, or hypertension (Kim et al., 2009). Some of the most important varieties in terms of protein quality are *Agaricus bisporus*, *Flamullina velutipes*, *Tricholoma matsutake*, and *Pleurotus eryngii* (Gao et al., 2012; Zhang et al., 2017).

Poddar et al. (2013) studied the effects of consuming white mushrooms and meat on individuals who entered a weight loss program and found a reduction in initial body weight of 3.6% for the group that consumed mushrooms, compared to those who consumed meat, who lost 1.1% of their body weight. Wang et al. (2019) used *Lentinula edodes* as a partial analogue of lean pork to obtain sausages and found that the resulting products had improved physicochemical and sensory properties.

Stephan et al. (2018) made vegetarian sausages from *Pleurotus sapidus*, obtaining products with physicochemical properties similar to traditional sausages, with a similar texture, but with better sensory value than other vegetable proteins. Making beef burgers with 5-15% *Agaricus bisporus* mushrooms to reduce fat content resulted in products with good sensory properties, good tenderness, and high oxidative stability (Patinho et al., 2021).
Soy flour contains an excellent amino acid profile and is widely used as a major indispensable protein in animal industries. This vegetable has a high content of protein (33-49%), fats (15-26%), non-nitrogenous extractive substances (13-24%), vitamins (A, B1, B6, D, E, K) and enzymes (lipoxidase, lipase, urease, amylase). Chen et al. (2010) investigated the nutritional quality of soy flour fermented with *Aspergillus* and *Lactobacillus*, demonstrating a significant improvement in soluble proteins in trichloroacetic acid, in vitro protein digestibility, and available lysine content, especially in the case of fermentation with Lactobacillus. They also produced a large amount of lactic acid, resulting in a lower pH compared to unfermented soy flour or soy protein concentrate ($p<0.05$).
Chickpeas present a several health benefits, being an important source of proteins (18.4 - 29%), lipids (4.5-6%), fibers (4.3-17.4%), carbohydrates (59.5-69.4%) and mineral salts (2.48-3.5%) (Boye et al., 2010). Phenolic compounds from chickpeas, such as Enterodiol, Gomisin D, Anhydro-secoisolariciresinol, Pelargonidin 3,5-O-diglucoside, Hesperetin 3′,7-O-diglucuronide, 6-Geranylnaringenin, Isorhamneti, p-Coumaroyl glucose, hydroxytyrosol 4-O- glucoside, confers antioxidant activity and can prevent degenerative diseases or can be cancer inhibitors (Perez-Perez et al., 2021). Chickpeas are also an important source of polyunsaturated fatty acids (66%) and unsaturated (19% of total fat) (Madurapperumage et al., 2021), which helps to reduce cholesterol, obesity and diabetes (Achari and Jain, 2017).
Obtaining pork meatballs with 30% LM-TVP or HM-TVP from peas, pumpkin or sunflower has been found to result in a slight decrease in protein quality compared to pure meat products, but also an increase in the quantity of mono and polyunsaturated fatty acids, as well as an increase in dietary fiber content (Baune et al., 2022).
More, an increase in dietary fiber content was reported for beef meatballs, in which 10-40% of the beef was replaced with rehydrated LM-TSP, while the total moisture and fat content was lower (Bakhsh et al., 2021).

CONCLUSIONS

According to studies, vegetable derivatives are widely used in the meat industry with positive effects on quality and often with a sensory value close to the meat products or at least accepted by consumers. In addition to nutritional advantages, another important aspect is their accessibility and relatively low price, which allows for sustainable exploitation of resources to ensure food production. The use of raw materials or plant derivatives for the development of new products (meat or other food) is an evolving field that is of interest to scientists and can be further studied.

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METHODS OF MICROSCOPIC SLIDES PREPARATION TO IDENTIFY THE POLLEN GRAINS DERIVED FROM DIFFERENT BEE PRODUCTS

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Abstract

Palynological research has applicability in several scientific fields, the most important being taxonomy, plant evolution, medicine and the analysis of honey - melissopalynology. The palynology provides data regarding the botanical and geographical origin of bee products like bee pollen, bee bread, royal jelly and propolis, which is useful in establishing their provenance and correct labelling. This paper details the characteristics and investigation of several methods for obtaining microscope slides from different bee products. The microscopic examination represents an essential part of the palynological analysis. Several methods for the preparation of microscopic slides were selected and assessed regarding preparation time, costs, dangerous substances used and results. The methods were employed successfully for different bee products, but the non-acetolysis method published by Louveaux et al. (1978) offers several advantages in terms of ease of use, safety and efficiency. The results of our comparative analysis emphasize that some methods are recommended for the creation of reference libraries, while others are well suited for being used in routine analysis of bee products.

Key words: *bee products labelling, microscopic analysis, palynology, slide preparation.*

INTRODUCTION

Palynology is the science that deals with the study of pollen and spores, both viable and fossil. The most important object of study is the pollen grains, which are analyzed morphologically and botanically (Grant-Downton, 2010). The term palynology was coined by English botanists, Hyde and Williams, in 1944. "Palynology" is a term derived from two Greek words, "*palinos*" meaning "powder/fine particles" and "*logos*" meaning science. The study of pollen focuses on its morphological details, polarity, symmetry, size, shape and openings on its surface ("General Botany, Botany Subdisciplines, Palynology," 2019) (www.biocyclopedia.com, 2019).

The results find applicability in many fields such as plant taxonomy and evolution, ecology, beekeeping, plant culture, archaeology, medicine, entomology and forensics (Jarzen, 2023).

Another branch and one of the most important of palynology are melissopalynology. It deals with the study of pollen sediments in honey with the aim of identifying the botanical and geographical origin of the honey. It has a decisive role in the price of honey, establishing the name under which it can be sold and subsequently how the lots will be labelled. Following the microscopic analysis, the botanical species with the predominant pollen from the honey sample is determined (Ige & Obasanmi, 2014). It is checked whether or not it is an attempt at food fraud (Soares et al., 2017) or whether the composition contains *Echium vulgare* pollen, a species declared toxic, due to the alkaloids in its composition (Dübecke et al., 2011).

Palynology is a tool used to give the botanical and geographical origin information of bee products like bee pollen, bee bread, royal jelly and propolis (Rojo et al., 2023).

Many beekeepers currently rely heavily on the bee pollen trade as a significant source of revenue. Bee pollen's botanical origin has a significant role in defining its quality and is extremely important to its price. Therefore, it is especially crucial to standardize the procedures used to gather this data with accuracy. For assessing the botanical profile, there is a fair number of research and a variety of methodologies utilized. The criteria used to categorize bee pollen as mono or multifloral is made in a lot of modes. The latter is particularly significant for businesses since it affects the bee pollen market price (Almeida et al., 2022).

In the case of propolis, the palynological analysis gives information regarding the botanical origin, the flora that can be found in the area during the picking and the difference between propolis (a product elaborated by honey bees (*Apis*) and geopropolis (a product elaborated by stingless bees) (Barth & Freitas, 2015).

Regarding RJ (royal jelly), palynological studies are useful for determining which plant species and vegetation types are of interest to bees (Barth, 2005), but the important for marketing is the determination of the botanical origin (Yil et al., 2022) or more correctly the determination of the pollen content of this beekeeping product.

Microscopic examination constitutes the essential part of the palynology analysis assessing both overall aspects (polarity, shape, color, apertures) as well as details (morphology of the exine and its ornamentation) of pollen grains. Several methods for the preparation of microscopic slides were selected from different references and assessed regarding preparation time, costs, dangerous substances used and results.

MATERIALS AND METHODS

A. Methods of obtaining palinotheque from freshly collected pollen

Sampling pollen from inflorescences

When collecting the pollen from the inflorescence, the samples are placed in plastic or paper envelopes and marked accordingly. In the case of unknown species, it is necessary to take a larger sample for further identification (Secomandi, 2018). Depending on the size and morphology of the flower, the entire stamen (Figure 1.a), individual anthers (Figure 1.b) or the entire flower (Figure 1.c) can be harvested.

I. Direct method

The gelatin-glycerin is dissolved on the induction hotplate at a temperature of 40ºC. A drop is placed on the slide, over which, with the help of a spatula, the pollen from the surface of the anther is transferred and homogenized with glycerine (Figure 2). Finally, a lamella is placed over the preparation.

Microscopic preparations made by this method contain fresh pollen with a viable cytoplasmic interior. For the microscopic analysis to be as precise as possible, a sufficiently large amount of pollen grains must be found on the slide so that they can be analyzed from all plans (Kisser, 1935; Secomandi, 2018).

II. Ether-based method

The present method requires particular materials and instruments, namely: watch glass, ethyl ether, pipettes, distilled water, tweezers, a cover and an induction hotplate. The working protocol according to (Secomandi, 2018) is presented in the following paragraphs.

The anthers or whole flowers are placed a watch bottle marked with an identification code or number and species name. Over the anthers, ether is added over the anthers until complete evaporation in the chemical niche, as shown in Figure 3.

Afterward, the remains of the anther are removed; distilled water is added and homogenized with a Pasteur pipette.

A drop of the resulted solution is taken and placed on the slide in a square shape that fits the lamella's dimension. The slide is left on the hotplate at a temperature of 40ºC, a drop of gelatin-glycerin is added and finally the lamella is placed over the preparation.

III. Acetolysis method

The acetolysis method was first published by (Erdtman, 1943) and is frequently used today (Hesse & Waha, 1989) It has undergone numerous improvements or modifications over time: (Lieux, 1980; Louveaux et al., 1970; Louveaux et al., 1978; Martins d'Alte, 1951; Maurizio, 1953).

Figure 1a. Sampling of the entire stamen

Figure 1b. Sampling individual anthers
by sectioning of the flower

Figure 1c. Sampling the entire flower

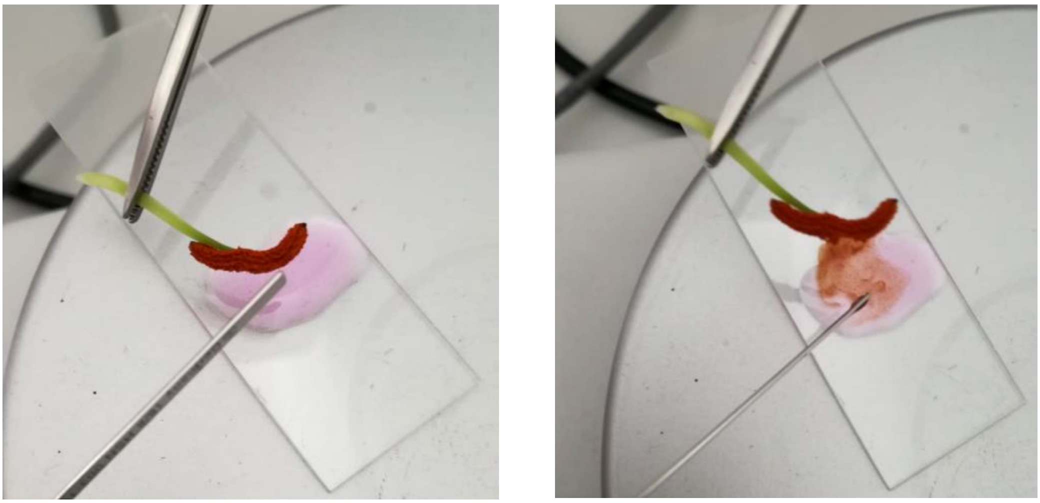

Figure 2. Preparation of microscopic slides based on gelatin-glycerin method

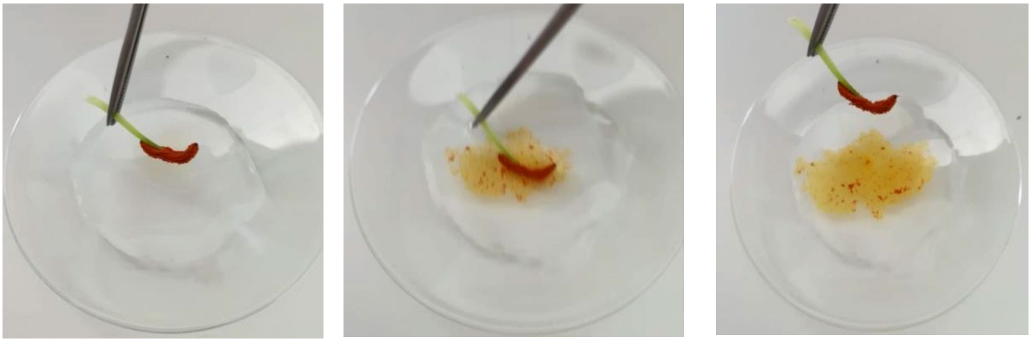

Figure 3. Preparation of ether-based microscopic slides

Figure 4. Required steps of the method without acetolysis

Acetolysis involves the dissolution of most tissues, organic debris and removes proteins, lipids and carbohydrates from the surface of pollen grains (Jones, 2014), leaving only pollenin, the material from which the exine of pollen grains is formed.

By completely removing non-pollen debris, such as intine, cytoplasm and genetic material, the grain structure is damaged and limits pollen analysis, changing morphology, shape and structure (Hesse & Waha, 1989).

A working protocol has been developed by (J. Louveaux et al., 1978) accordingly.

Anthers are mixed with 50 mL of warm distilled water (i.e. < 40ºC), and mix centrifuged for 10 minutes at 2500 rpm. Afterward, the remains of the anthers are separated and the supernatant is removed with a Pasteur pipette, with a final solution of 1 mL remaining.

Distilled water is added over the sediment, homogenized and transferred into two 10-20 mL micro centrifuge tubes in equal parts and finally centrifuged for 5' after which the supernatant is separated. The remaining sediment is drained on a filter paper and left to dry. The second tube is separately placed.

Afterward, 10 mL of acetolizing mixture is prepared by adding H_2SO_4 and acetic anhydride (1:9). The used instruments need to be thoroughly well dried (Caution: Acetolysis fluid reacts quite vigorously with water and causes bubbling and splashing).

A drop of acetolytic mixture is added to the dried sediment. The sediment is homogenized thoroughly using a glass rod along with the remaining mixture. The tube is placed on a water bath at a temperature of 70°C, taking into account that the water will not come into contact with the acetolytic agent. After an incubation period of 5 minutes, the tube is centrifuged. The supernatant is transferred into a dry vessel and the amount of acetolyzed pollen is visibly noticeable lower compared with the amount from the second tube.

The tube is filled with distilled water and a drop of detergent and mixed vigorously and centrifuged once more for 5 minutes.

After the removal of the liquid supernatant, an important aspect is to observe the presence of pollen grains to the walls. In this case the washing procedure must be repeated with an increased centrifugation at 3500 rpm.

The entire sediment is placed on a glass slide in a 20 x 20 mm surface using a Pasteur pipette.

The blade is dried on a magnetic hotplate at 40ºC.

If the resulting sample contains a small amount of pollen, the acetolization process will be carried out for the second tube as well and afterward the obtained sediment quantities will be combined.

Acetolysis of pollen must be performed in a hood under laminar flow and laboratory equipment is mandatory, because the acetolytic mixture (consisting of sulfuric acid and acetic anhydride) is corrosive and reacts violently in contact with water (Jones, 2014).

IV. The rapid method with acetolysis

The quick and easier-to-implement variant of the previous method is to use the acetolizing mixture directly on the anthers of the flowers, placed on a watch glass. These are mixed and incubated until the pollen separates from the anther tissues, which are then removed. The rest of the mixture is transferred to a slide and dry it under the flame of a spirit bottle, avoiding contact with the direct flame, to prevent excessive blackening of the pollen. After complete evaporation, with the help of a spatula, the pollen is transferred to a new slide where a drop of glycerine is added (Grant-Downton, 2010; Halbritter, 2018).

V. Method without acetolysis

The method without acetolysis, according to the protocol described by (J. Louveaux et al., 1978) is as follows and presented in Figure 4.

A minimum of 7-8 harvested anthers are inserted into each Falcon tube and 10 mL H_2SO_4 5‰ is assed. The samples are centrifuged for 10 minutes at 3500 rpm. After centrifugation, the supernatant is harvested and discarded, double-distilled water is added and after vigorous shaking it is re-centrifuged, followed by removal of the supernatant.

Using a Pasteur pipette, the complete amount of pollen is collected which is present at the base of the tube in the form of sediment.

The collected sediment is placed on the microscope slide on a 20 x 20 mm surface, dried on an induction plate (that must not exceed 40ºC) or directly at room temperature.

B. Melissopalynology

The method is used as a tool for the determination of the botanical and geographical origin of honey.

Slide preparation without acetolysis

The protocol described by (Louveaux et al., 1978) is as follows. A quantity of 20 mL of distilled water which is not exceeding 40°C is used to dissolve 10 g of honey. The honey sample is dissolved with diluted sulphuric acid or diluted potassium hydroxide (5 g H_2SO_4 or 100 g KOH to 1 liter of water) in cases of honey samples high in colloidal particles. The liquid supernatant is discarded from the solution after centrifuging for 10 minutes at roughly 2500 r/min. For a thoroughly removal of honey sugars, the previous step is repeated, accordingly; the sediment is mixed with approximately 10 mL of distilled water and centrifuged for 5 min. The whole amount of the sediment is spread out over a slide in a 20 mm x 20 mm. In most cases it is recommended to use the Pasteur pipettes for transferring the sediment from the centrifuge tube to the slide. The sediment is mounted after drying with glycerine gelatine (optional fuchsine) which has been liquefied by heating in a water bath at a temperature of 40°C.

Slide preparation with acetolysis

The sample weight is the same as the previous method (e.g. 10 g) and the steps are presented in section A.III. Methods of obtaining palinotheque from freshly collected pollen, acetolysis method.

C. Bee pollen and bee bread analysis

The bee pollen and bee bread samples must be ground and well homogenized, following the protocol described by (Louveaux et al., 1978), with slight modifications. According to (Almeida-Muradian et al., 2005) the sample to be analyzed must weigh 2 g. The initial suggested approach underwent various modifications to increase repeatability within and across laboratories and to evaluate some of the probable factors that could generate the variability of the findings obtained. According to preliminary findings, the pollen sample size must be increased to 5 g in order to improve its representativeness (Almeida et al., 2022). The steps followed in preparing the microscopic preparation are the same as for the honey sample (see Section B. Melissopalynoloy).

D. Pollen analysis of royal jelly (RJ)

The slide preparation to assess the qualitative analysis of RJ is realized after (d'Albore & Bernardini, 1978; Von Der Ohe et al., 2004).

A conical-bottom centrifuge tube of 50 g is used after 1 g of RJ is weighted with the addition of 10 mL of KOH 1% and vortexed. The sample is centrifuged for 10 minutes at 2500 rpm and the supernatant is discarded. A quantity of 10 mL distilled water is added, thoroughly (vortex) mixed, following the complete fill of the test tube with the remaining distilled water and reaching a total volume of about 45 mL. The resulting mixture is centrifuged for a second time for 10 minutes at 2500 rpm and the finally the supernatant is discarded. The sediment is transferred from the centrifuge tube to the slide drawing this time a much smaller surface (10 x10 mm), compared to the one from the honey analysis.

A different technique performed by (Barth, 2005) involves the dilution of a quantity of 1g of RJ in 10 mL of distilled water with the addition of a single tablet of alien spores (*Lycopodium clavatum*; 10679 spores per tablet) to the mixture (Stockmarr, 1971). The sediment is centrifuged, re-suspended in 10 mL of acetic acid, and allowed to sit overnight to dehydrate. The sediment is acetolyzed in the following day (see the method A.III.)

E. Pollen analysis from propolis

Propolis

The protocol proposed by (Barth, 1998) as follows: a quantity of 0.5 g of the scraped propolis extract is mixed with 15 mL of ethanol overnight or for a couple of days with occasional shaking of the tube. The suspension is divided into two tubes and centrifuged. After centrifugation the decanted sediment is re-suspended with 13 mL ethanol. Over the sediment 12 mL of KOH (10%) is added and brought to boiling point for at least 2' in a water bath. After this step, the tubes are placed in an ultrasonic agitator for 5', centrifuged and decanted. A quantity of 13 mL of distilled water is added, the sediment is transferred to another centrifuge tube, throughout a 0.3 mm mesh sieve for the removal of large organic particles, followed by centrifugation, decantation. Finally, a slide of the sediment is prepared using glycerine-jelly.

The use of acetolysis in other methods requires the following steps. Over the propolis sample a quantity of 5 ml of glacial acetic acid is added and left overnight. Afterward, the sample is centrifuged and decanted, and the acetolysis mixture composed of acetic anhydride and concentrated sulphuric acid (9:1) is added. The mixture is left for 3 minutes at 80°C in a water bath, centrifuged and decanted. The resulting sediment is washed with distilled water and after a mixture of glycerine and water (1:1) is added and left to rest for at least 30', followed by centrifugation and decantation of the sediment. The final slides are prepared, one with unstained glycerin jelly and another with basic fuchsin-stained glycerine-jelly.

RESULTS AND DISCUSSIONS

Due to their beneficial effects on human health, apicultural products have been utilized in phytotherapy and nutrition for a long time. The presence of bioactive chemicals has been thought to provide health benefits, they are becoming more popular nowadays (Ares et al., 2018). The bioactive compounds are in close correlation with the botanical and geographical origin. Due to the growing consumer desire for distinctive products with a sense of place, the use of geographical indication (GI) for bee products is necessary to obtain consumer confidence, leading to market recognition and a premium price (Sattler et al., 2015).

By the use of palynological or melissopalynological examination, the bee product can be labelled according to its botanical and/or geographic origin. The quality of bee products is correlated with their botanical and geographic provenance, which can serve as indicator in their identification of origin and labelling (Mădaş et al., 2020).

Since palynological analysis often gives a subjective result, it must be complemented or correlated with other determinations that justify the obtained results. Among the quality criteria Bogdanov assessed several constituents, including: electrical conductivity, reducing sugars, sucrose concentration, free acid, proline and protein content, hydroxymethylfurfural (HMF), and diastase activity (Bogdanov et al., 2002). Additionally, other quality markers used are color, phytochemicals, and sensorial characteristics of honey (Thrasyvoulou et al., 2018).

To define honey from different areas or to detect a mixture of honey from different locations some researchers, have established a model for predicting the geographic origin of honey using pollen grains frequency from the same type of honey samples produced in different areas or combining this strategy with cluster and correlation statistical analysis was effective for describing honey samples of different geographical and botanical origins (Herrero et al., 2002; Karabournioti et al., 2006).

The melissopalynological analysis may be influenced by the flower's morphology and physiology (Louveaux et al., 1978; Todd & Vansell, 1942), but also according to the foraging bee activity or hive contamination (Molan, 1998). There are several unifloral honey types with underrepresented pollen, such as Citrus, Lavandula, Rosmarinus and Salvia between 10 and 20%, and Tilia, Medicago, and Robinia with pollen grains between 20 and 30%. On the contrary, Brassica (>60%), Castanea (>90%) and Eucalyptus (>80%) honey types contain overrepresented pollen (Von Der Ohe et al., 2004). In this context, mellisopalynology coupled with other analysis may provide reliable results in terms of authenticity and discrimination of honey samples (Corvucci et al., 2015). a number of honey sample quality variables were examined in relation to the prevalence of overrepresented pollen grains. Several methods were examined, mainly: melissopalynological, organoleptic, physicochemical (water content, electrical conductivity, color), and volatile properties of the blends of overrepresented mixed honeys, specifically eucalyptus and chestnut honeys with thyme honeys in various analogies. The most sensitive metrics were microscopic features, followed by organoleptic (Rodopoulou et al., 2018).

The combination of these analysis with multivariate analysis techniques (e.g. hierarchical cluster analysis (HCA) and principal component analysis (PCA)) provides a more precise classification of the honey samples (Geana & Ciucure, 2020). Authenticity of multiple monofloral and multifloral honey samples has been assessed using the method described by Louveaux without acetolysis (Louveaux et al., 1978), along with SDS-PAGE and combined with multivariate analysis. This novel methodology has been evaluated to provide a simple, low-cost method for identifying honey sources, as well as new opportunities to detect honey adulteration. The generated heatmap depicted the link between melissopalynological analysis and protein pattern providing a reliable method in honey authentification (Mureșan et al., 2022).

The method with acetolysis has been successfully used to characterize the pollen grains from different species of the genus Anthemis (Dauti et al., 2014). In a different study, the same method was implemented to evaluate the differences in palynomorphological characteristics of different plants found in different areas in Albania. Furthermore, the colored method of basic fuchsine and fixation with gelatine-glycerin were also used (Kallajxhiu et al., 2015).

The method without acetolysis firstly introduced by (Erdtman, 1943) and modified by (Louveaux et al., 1978) was used to assess the foraging preferences of bees from different tropical regions. In the context of plant-pollinator interactions, this study determined the variations in pollen contents of honey across time and distance, even within the restricted landscape mosaic (Ponnuchamy et al., 2014).

The mellisopalynological analysis using the ether-based method was evaluated to recognize the pollen granules of Mediterranean floral species from multiple honey samples. In order to evaluate the pollen grains in their natural color, which can vary depending on the botanical family, microscopic observations were first done without the addition of any coloring additives. A colored preparation with fuchsin was used in order to improve the mellisopalynological recognition. Furthermore, in order to perform a more thorough study DNA extraction was also carried out. The microscopic analysis of the pollens removed from certain commercially available unifloral honeys was supported by the results of the molecular study, which was conducted using RT-PCR. The microscopic examination revealed the presence of pollen grains from both the species listed on the honey label and other species. It was possible to conduct an analysis on the pollen composition of some honey that was marketed and designated unifloral due to the application of microscopic and biomolecular analysis techniques in melissopalynology (Vancheri et al., 2019).

In a different study, the techniques reported by (Almeida et al., 2022; Louveaux et al., 1978; Morais et al., 2011) modified for pollen load formed the basis of palynological study (Margaoan et al., 2014). The palynological analysis was complemented with individual carotenoids and fatty acids identification. The levels of lipids and carotenoids in bee-collected pollen varied greatly between samples, which can be explained by the different botanical sources of those samples.

CONCLUSIONS

The findings of our comparative study demonstrate that while some strategies are recommended to be used in the development of reference libraries, while others are best suited for use in bee product analysis. A high level of proficiency in both microscopy and botany are necessary for palynology analysis. Furthermore, the use of different methods to identify the pollen types and characteristics are significant to assess the origin and even authenticity of the samples when coupled with other analysis.

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ASSESSING THE ABILITY OF FREEZE-DRIED EXTRACTS FROM BLACKBERRY PROCESSING BY-PRODUCTS TO ENHANCE THE ANTIOXIDANT FUNCTION OF SUNFLOWER OIL DURING HIGH-TEMPERATURE HEATING

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Abstract

The aim of this study was to assess the ability of two freeze-dried extracts from blackberry processing by-products resulted after juice extraction, compared to butylhydroxytoluene (BHT) in preventing the lipid oxidation of sunflower oil (SFO) subjected to high-temperature heating at 180℃ up to 12 hours. The blackberries were collected from spontaneous flora of two regions of Romania, Zugau (Arad County) and Paltinis (Sibiu County) and the blackberry by-products extracts (BBE) were noted according to the place of origin as ZBBP, respectively PBBE. The evolution of lipid oxidation was tracked by means of specific indices as: peroxide value (PV), p-anisidine value (p-AV), total oxidation (TOTOX) value and the response of TBA-malondialdehyde (MDA) interactions evaluated by thiobarbituric acid (TBA) method. The results revealed that BBE displayed a high inhibitory effect on SFO thermo-oxidation. This response was dose-dependent, thus, the rate of lipid oxidation was inversely related to BBE concentration. The recorded data highlighted that BBE constitutes efficient natural antioxidants that can contribute to the development of sunflower oil with extended thermo-oxidative stability.

Key words*: blackberry processing by-products, freeze-dried extracts, inhibitory effect, lipid oxidation, thermo-oxidative stability.*

INTRODUCTION

Sunflower oil (SFO) is one of the top-quality and most widely used vegetable oils in industrial applications as well as in human nutrition, due to its composition of up to 85% unsaturated fatty acids, especially linoleic essential acid, and to its very high burning point (El-Hamidi & Zaher, 2018; Metzner Ungureanu et al., 2020c; Orsavova et al., 2015).

However, during food processing, due to the prolonged exposure of SFO to high temperatures, various chemical reactions such as thermal oxidation, polymerisation and hydrolysis take place, which produce changes in the physico-chemical properties and nutrient content (Abd Razak et al., 2021; Ramroudi et al., 2022).

The electric convection oven is an universal heat source, used in various thermal food applications. The main cause of the SFO degradation during convective heating, is lipid oxidation that involve a wide range of complex chemical reactions followed by the formation of primary oxidation compounds (Poiana et al., 2022). From primary oxidation compounds, secondary oxidation compounds will be developed, and so forth for tertiary compounds or further oxidation compounds. In recent years, emphasis has been placed on replacing the synthetic antioxidants previously used to improve the oxidative stability of SFO subjected to thermal processing, due to their damaging potential, with healthy, natural alternatives (Lorenzo et al., 2017). Therefore, the detection of new natural sources of bioactive compounds with antioxidant potential, to replace synthetic additives such as butylhydroxytoluene (BHT), represent a priority in food industry (Fadda et al., 2022; Grebenteuch et al., 2021; Poiana et al., 2021; Raba et al., 2020).

Different studies have proved the potential of vegetable waste such as berry processing by-products, as sources of natural bioactive compounds with antioxidant activity due to their high phenolic content (Metzner Ungureanu et al., 2020b; Moraru Manea et al., 2022; Zeng et al., 2023). Berry processing by-products represent low-cost extraction material for natural antioxidants suitable in preventing food and oils lipid oxidation (Metzner & Poiana, 2018; Popa et al., 2011).

Antioxidant properties in terms of antioxidant activity, total phenolic content and individual polyphenolic compounds profile of the extracts obtained from different fruit processing by-products, have been previously exposed by methods specific to those type of investigations (Kuppusamy et al., 2020; Lyu et al., 2023; Metzner Ungureanu et al, 2020b).

For the most part, the impact of berry extracts on lipid oxidation, has been studied on meat products (Burri et al., 2020; Ganhão et al., 2013; Varzaru et al., 2020). Therefore, Ganhão et al. (2013), on their research on several Mediterranean berries, reported that the berries extracts were capable to inhibit the lipid oxidation in raw, cooked and cooked and chilled porcine burger patties and Varzaru et al. (2020) revealed that extracts resulted from bilberry, cranberry and raspberry leaves exposed an inhibitory response on lipid peroxidation on chickens meat. Also, Burri et al. (2020) demonstrated that bilberry and red currant extracts, are efficient against lipid oxidation developed in a processed meat model system.

Although multiple studies recommend the exploitation of fruit processing by-products as reliable sources of bioactive compounds with antioxidant activities, so far, there are only a few data about the inhibitory response of berries processing by-products in limiting the lipid thermo-oxidation.

Taking into consideration the mentioned data, the purpose of this research was to assess the effect of freeze-dried extracts obtained from blackberry processing by-products resulted after juice extraction, to inhibit the lipid oxidation of SFO subjected to high-temperature convective heating in order to substitute BHT. Thus, the aim was to determine the most effective extract, the most efficient dose, and the impact of the origin area of the blackberries on the antioxidant properties of freeze-dried extracts, in order to obtain a superior inhibition of SFO thermo-oxidation. In this regard, the evolution of lipid oxidation initiated by the exposure of SFO to convective heating at 180ºC for different periods of time, was evaluated by means of specific indices as: peroxide value (PV), *p*-anisidine value (*p*-AV), total oxidation (TOTOX) value and the response of TBA-malondialdehyde (MDA) interactions was evaluated by thiobarbituric acid (TBA) method.

MATERIALS AND METHODS

Processing of blackberry by-products extracts

Blackberries (*Rubus fruticosus* L.) were collected at maturity stage in the year 2018, from two different Romanian areas: Zugau, Arad County and Paltinis, Sibiu County. Compared to the Paltinis area, Zugau area present a milder climate with moderate precipitation regime and higher temperatures. After juice extraction, resulted blackberry by-products were conditioned in an electric oven with convective heat (Esmach SpA-Ali Group/Italy, 1200 W, 50 Hz) at 60ºC for 12 h. Conditioned by-products were grinded using a laboratory mill (Grindomix Retsch GM200, Germany) and the extraction of bioactive compounds was completed by a maceration solvent extraction in a hydro-alcoholic ethanol/water mixture (1:1, v/v) where the solid:solvent extraction ratio was 1:10 (w/v), for 48 h at 20ºC. After percolation, the clear extracts resulted were evaporated until 100 mL, using a rotary evaporator (RV 10 auto pro V, IKA England Ltd., 100 W, 50 Hz). Further, the blackberry by-products extracts were subjected to freeze drying using a lyophilizer (Alpha 1-2 LD plus, 230 V/50 Hz, MARTIN CHRIST GmbH, Germany). According to the origin place of blackberries, the freeze-dried extracts obtained were noted as ZBBE for Zugau area, respectively PBBE for Paltinis area.

Application of freeze-dried blackberry by-products extracts to SFO

Freshly refined and free of synthetic antioxidants SFO was divided into eight portions. Six portion were supplemented with 200, 500 and 800 ppm freeze-dried blackberry by-products extracts (ZBBE and PBBE), the seventh portion was supplemented with 200 ppm BHT and the last portion of SFO, without any additive, was used as a control (C). For a better diffusion of supplements in SFO, before applying, the extracts and BHT were diluted in a minimum volume of absolute ethanol in an ultrasonic water bath and then were mixed with SFO for 10 min, followed by a vacuum evaporation. The control sample was prepared in identical conditions.

High-temperature heating of SFO

In order to evaluate the inhibitory effect of freeze-dried extracts and BHT on thermo-oxidative lipid degradation in simulated frying conditions, the SFO samples were exposed to a temperature of 180ºC for 3, 6, 9 and 12 h using a convection electric oven (Esmach SpA-Ali Group/Italy, 1200 W, 50 Hz). For each investigated sample 25.0 ± 0.5 g of SFO was weighted. Separate oil samples were used for different heating period.

Freeze-dried blackberry by-products extracts (BBE) analysis

Moisture content: The moisture content of the BBE was determined according to the method 925.09 of the AOAC (Horowitz & Latimer, 2006).

Total phenolic content (TPC): The TPC of BBE was spectrophotometrically assessed using the Folin-Ciocalteu method, as it was previously reported by Metzner Ungureanu et al. (2020c) and the results were expressed as mg gallic acid equivalent (GAE)/g dried substance (d.s).

Antioxidant activity (AA): The AA has been investigated with the DDPH assay by which the free radical scavenging ability of BBE was tested using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the previously described method. The results were expressed as mg GAE/100 g d.s. and the inhibition of DPPH was expressed as percentage (Metzner Ungureanu et al., 2020b).

The polyphenolic compounds profile: The individual phenolic compounds of BBE were identified by chromatographic analysis using an UHPLC (ultra-high-performance liquid chromatograph) and the results were expressed as mg/100 g d.s (Metzner Ungureanu et al., 2020b).

Evaluation of lipid oxidation

The peroxide value (PV): The PV has been evaluated by the iodometric titration according to the standard methods for oils analysis and the results were expressed as milliequivalents of active oxygen per 1000 g oil sample (mEq O2/kg oil). PV represents a reliable indicator to assess the occurrence of primary lipid oxidation (AOCS, 1998).

The p-anisidine value (p-AV): The *p*-AV has been spectrophotometrically determined according to the standard methods for oils analysis and it provides information about the occurrence of secondary products of lipid oxidation (AOCS, 1998).

The total oxidation (TOTOX) value: TOTOX value reflects the contribution of both PV and *p*-AV to the total lipid oxidation and it was obtain by the following equation: TOTOX= 2PV+ *p*-AV (Esfarjani et al., 2019; Metzner Ungureanu et al., 2020c).

The thiobarbituric acid (TBA) assay: the TBA value was assessed spectrophotometically according to the slightly modified procedure of Singh et al. (2006) in agreement with the method reported by Metzner Ungureanu et al. (2020c) and the results were expressed as µg MDA/mL oil sample. The TBA assay depends by the reaction of MDA (malondialdehyde) with TBA and is used specifically in the evaluation of lipids secondary oxidation (Zeb & Ullah, 2016).

Statistical data analysis

All determinations were performed in triplicate and the results were reported as mean values ± standard deviation (SD). One-way ANOVA analysis of variance was used for statistical data analysis. Computations Tukey post-hoc means comparisons and Levene's test for equal variance were included to estimate the statistical significance of variations among means. Data within the same row or column for tables, or bars, for charts, sharing different letters are significantly different ($p < 0.05$).

RESULTS AND DISCUSSIONS

Freeze-dried blackberry by-products extracts (BBE) analysis

Data exposed in Table 1 reveal the TPC of BBE and their antioxidant activity evaluated by the DPPH assay.

The residual moisture content of ZBBE and PBBE was determined in order to express the antioxidant characteristics relative to dried substance (d.s) content. The recorded moisture content was low in both ZBBE (3.18%) and PBBE (3.56%). Effective reduction of the humidity make the microbial and enzymatic degradation impossible, thus conferring a high shelf life.

Table 1. Antioxidant properties of freeze-dried extracts obtained from blackberry processing by-products

Sample	TPC (mg GAE/ g d.s)	DPPH	
		I (%)	(mg GAE/ 100g d.s)
ZBBE	105.34±0.54^{a}	92.84±0.51^{a}	1375.14±8.05^{a}
PBBE	76.13±0.38^{b}	90.82±0.44^{b}	1044.52±6.32^{b}

The results showed significant differences between the antioxidant characteristics of the freeze-dried extracts investigated. Thus, the TPC values recorded were 105.34 in ZBBE and 76.13 mg GAE/g d.s in PBBE. It is noted that the TPC value was 38% higher in ZBBE compared to the PBBE sample. Piasecka et al. (2022) reported a TPC value ranging between 48.28 and 50.16 mg GAE/g in extracts obtained from wild flora blackberry processing by-products dried in a desiccator under vacuum.

In terms of AA expressed as mg GAE/100 g d.s, a loss of 24% was noted in PBBE sample compared to ZBBE. The freeze-dried extract obtained from blackberry processing by-products from the Zugau area showed higher antioxidant attributes compared to those recorded for PBBE, therefore a milder climate results in a higher TPC and a superior AA.

Most polyphenolic compounds identified in BBE after UHPLC analysis, were consisted in phenolic acids as: p-coumaric acid, caffeic acid, rosmarinic acid, vanillic acid, gallic acid and syringic acid. Other polyphenolic compounds identified in freeze-dried extracts obtained from blackberry by-products were rutin and pyrocatechol. Of all polyphenolic compounds identified, pyrocatechol was recorded in the highest amount. The data obtained from the chromatographic analysis, correspond to the data reported by Jazić et al. (2019) for blackberry processing by-products.

Although the same polyphenolic profile was identified in freeze-dried extracts, the amount recorded varied significantly depending on the region of origin. Thus, the highest amount of polyphenolic compounds was noted in ZBBE, except for pyrocatechol and syringic acid which had higher values in PBBE. Therefore, the region of origin, in the context of climatic conditions, may affect the concentration pattern of polyphenolic compounds. This finding is in agreement with similar previous studies, which have shown that area of origin, along with other factors such as variety, ripening stage, amount of hydration, storage and conditioning conditions, can influence the dynamics of bioactive substances (Dróżdż et al., 2017; Metzner Ungureanu et al., 2020a).

Evaluation of lipid oxidation

Peroxide value (PV)

Hydroperoxides are chemical compounds produced as a result of primary lipid oxidation. They are unstable and prone to enzymatic and non-enzymatic degradation, resulting in a wide range of secondary oxidation products. The peroxide value increases with increasing hydroperoxide formation coefficient when the rate of decomposition of the hydroperoxides into secondary oxidation products is exceeded by the rate of their formation (Poiana, 2012).

Table 2 shows the PV variations following the exposure of oil samples to a temperature of 180ºC for different periods of time.

Table 2. The impact of SFO supplementation with BHT and freeze-dried extracts obtained from blackberry processing by-products, on the peroxide value, during heat treatment

Time (h)	PV (meq O_2/kg Oil)		
	C	BHT **200** ppm	
0	1.81±0.05^{a}	1.81±0.05^{a}	
3	11.64±0.47^{a}	8.12±0.26^{d}	
6	11.15±0.43^{a}	7.87±0.23^{b}	
9	10.96±0.41^{a}	7.53±0.25^{b}	
12	10.79±0.38^{a}	7.38±0.31^{b}	
Time (h)	PV (meq O_2/kg Oil)		
	ZBBE (ppm)		
	200	**500**	**800**
0	1.81±0.05^{a}	1.81±0.05^{a}	1.81±0.05^{a}
3	9.52±0.38^{b}	8.56±0.29^{d}	6.82±0.15^{e}
6	8.76±0.34^{b}	8.14±0.27^{c}	6.68±0.14^{d}
9	8.43±0.31^{b}	7.98±0.28^{b}	6.12±0.17^{c}
12	7.92±0.25^{b}	7.67±0.19^{b}	5.92±0.18^{c}
Time (h)	PV (meq O_2/kg Oil)		
	PBBE (ppm)		
	200	**500**	**800**
0	1.81±0.05^{a}	1.81±0.05^{a}	1.81±0.05^{a}
3	9.65±0.39^{b}	8.66±0.32^{c}	6.97±0.16^{e}
6	8.98±0.36^{b}	8.52±0.33^{b}	6.85±0.15^{d}
9	8.61±0.30^{b}	8.31±0.31^{b}	6.69±0.19^{c}
12	8.11±0.26^{b}	7.84±0.27^{b}	6.03±0.22^{c}

One-way ANOVA test was used to compare the means differences registered for each heating period among the oil samples supplemented with BHT and different doses of BBE, relative to C; data within the same row sharing different letters are significantly different ($p < 0.05$).

A significant increase in PV value was observed in the first stage of thermal processing of oil samples, and in the interval 3-6 h of heat exposure, the peroxide value starts to decrease indicating the decomposition of primary oxidation products represented by hydroperoxides, into secondary oxidation products, aldehydes and ketones. By the end of the 12 h heating interval, PV follows a downward pattern.

Reduced primary oxidation was noted in response to supplementation with BHT and freeze-dried extracts obtained from blackberry processing by-products. Significant differences ($p<0.05$) in peroxide value were recorded during the heating process between the control sample and the samples supplemented with 200 ppm BHT and different doses of BBE. The inhibitory effect against primary oxidation is conditioned by the concentration of the applied extract. Therefore, the extracts in a dose of 800 ppm showed the highest inhibitory response in the first phase of thermo-oxidation. The results obtained are consistent with data recorded in similar studies in which the inhibitory response of BHT and natural extracts with antioxidant potential against primary oxidation promoted by exposure to high temperatures, was evaluated (Metzner Ungureanu et al., 2020c; Poiana, 2012).

Supplementation of SFO samples with BHT and freeze-dried extracts obtained from blackberry processing by-products resulted in a 30% reduction of PV in oil sample supplemented with 200 ppm BHT, respectively with 41% and 40%, in samples supplemented with 800 ppm ZBBE and PBBE, compared to the control sample, after 3 h of high temperature exposure, at which time the highest PV value was recorded.

In the study of Poiana (2012) regarding the improvement of oxidative stability of SFO subjected to convective and microwave heating up to 4 h by supplementation with grape seed extract, a steady increase in peroxide value was reported throughout the heating interval. At the end of the heating process, PV decreased by 32% in the oil sample supplemented with 200 ppm BHT and with 39% in the samples supplemented with 800 ppm grape seed extract. Also, in the study conducted by Khor et al. (2019) in which the effect of heating palm kernel oil at a temperature of 180ºC up to 24 h was investigated, the highest peroxide value was recorded after 4 h of heat treatment, and after this interval the PV was significantly reduced.

BBE obtained from blackberry collected from the region with a milder climate showed a higher inhibitory effect compared to BBE obtained from blackberry originating in the area with low temperatures and high rainfall. The obtained inhibitory response correlates with the antioxidant characteristics of the investigated extracts.

During thermal processing, freeze-dried extracts obtained from blackberry processing by-products at a concentration of 200 ppm showed a lower inhibitory response compared to BHT, at 500 ppm the response was similar to that recorded in samples added with BHT, and at a level of 800 ppm, the inhibitory effect of freeze-dried extracts was superior to that exposed by BHT (Metzner Ungureanu et al., 2020c).

***p*-Anisidine value (*p*-AV)**

Under constant action of heat treatment, the primary products of lipid oxidation continue to decompose generating secondary oxidation products that affect sensory properties, being responsible for change of smell and flavour.
Table 3 presents variations of *p*-AV over the heating process, in additive SFO samples with 200 ppm BHT and various doses of BBE.

Table 3. The impact of SFO supplementation with BHT and freeze-dried extracts obtained from blackberry processing by-products, on the *p*-AV, during heat treatment

Time (h)	*p*-AV		
	C	BHT **200** ppm	
0	2.49±0.15^{a}	2.49±0.15^{a}	
3	47.86±2.07^{a}	40.17±2.28^{b}	
6	56.98±1.93^{a}	50.39±2.43^{a}	
9	62.27±2.21^{a}	54.67±2.17^{a}	
12	69.84±3.49^{a}	58.85±2.52^{b}	
Time (h)	*p*-AV		
	ZBBE (ppm)		
	200	**500**	**800**
0	2.49±0.15^{a}	2.49±0.15^{a}	2.49±0.15^{a}
3	45.12±2.98^{a}	41.89±2.53^{a}	38.34±1.92^{d}
6	54.05±3.04^{a}	50.85±2.21^{a}	49.21±2.33^{b}
9	58.64±3.11^{a}	56.14±3.20^{a}	53.12±2.59^{b}
12	64.28±3.15^{a}	58.92±2.81^{b}	55.79±2.64^{c}
Time (h)	*p*-AV		
	PBBE (ppm)		
	200	**500**	**800**
0	2.49±0.15^{a}	2.49±0.15^{a}	2.49±0.15^{a}
3	46.03±2.41^{a}	42.35±3.03^{a}	38.67±2.25^{c}
6	54.51±2.56^{a}	52.27±2.94^{a}	50.02±2.97^{b}
9	60.57±3.43^{a}	56.73±3.42^{a}	55.12±2.82^{a}
12	65.72±3.95^{a}	59.16±3.48^{b}	58.51±3.34^{b}

One-way ANOVA test was used to compare the means differences registered for each heating period among the oil samples supplemented with BHT and different doses of BBE, relative to C; data within the same row sharing different letters are significantly different ($p < 0.05$)

The *p*-AV has increased steadily throughout the heat treatment application. The high *p*-anisidine value implies the reduction of the smoke point which resides in quality decreasing of the investigated oil. In response to supplementation of sunflower oil with various doses of BBE and BHT, a significant ($p<0.05$) reduction in *p*-AV was noted compared to C.
After 12 h of heating at 180ºC, a decrease in *p*-AV compared to C of 6%, 15% and 16% was observed for the oil samples supplemented with PBBE in concentration of 200 ppm, 500 ppm and 800 ppm, respectively 8%, 16% and 20% for the oil samples supplemented with ZBBE in concentration of 200 ppm, 500 ppm and 800 ppm, and 16% for the samples supplemented with 200 ppm BHT. Therefore, the effect of the investigated extracts in inhibiting secondary oxidation, is dose dependent. At a 200 ppm concentration, freeze-dried extracts showed a lower inhibitory response than BHT, at 500 ppm a similar response, and at 800 ppm, the inhibitory effect of PBBE was identical to that reported for BHT, while for ZBBE it was 4% higher. Recorded data indicate that at a dose of 800 ppm, ZBBE is a potential substitute for BHT. The results obtained are in agreement with data recorded in similar studies investigating the improvement of oxidative stability of sunflower oil subjected to heat by addition with extracts obtained from fruit processing by-products (El-Hadary & Taha, 2020; Metzner Ungureanu et al., 2020c). El-Hadary & Taha (2020) in their study focused on the enhancing of some vegetable oils oxidative stability by the addition of various doses of natural extracts obtained from pomegranate peel, found that increasing the dose of extract resulted in a significant improvement of the inhibitory response in the secondary oxidation stage.

Total oxidation (TOTOX) value

In order to estimate the quality of oils by determining total oxidation, it is necessary to establish in advance the specific chemical indices of primary and secondary oxidation. As a characteristic index for monitoring the oxidation process of vegetable oils, the TOTOX value corresponds to the level of total lipid oxidation. Therefore, increasing the total oxidation value proves an increased degree of lipid damage (Metzner Ungureanu et al., 2020c).
Figure 1 presents TOTOX variations noted in oil samples supplemented with various doses of freeze-dried extracts obtained from blackberry processing by-products, and 200 ppm BHT, during the entire heating process.

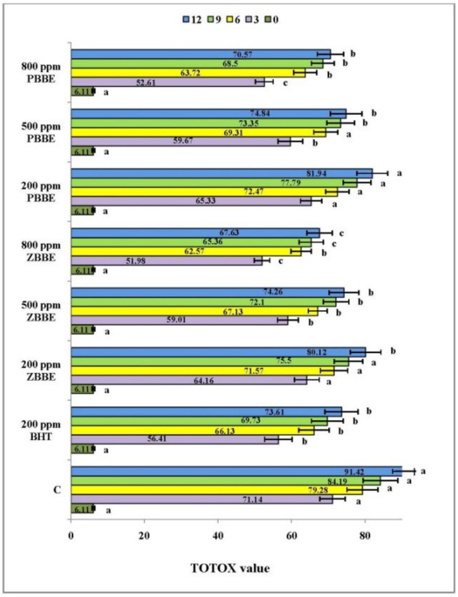

Figure 1. Effect of SFO supplementation with BHT and BBE on TOTOX value during high-temperature heating. One-way ANOVA test was used to compare the means differences registered for each heating period among the oil samples supplemented with BHT and different doses of BBE, relative to C; the values for bars sharing different letters are significantly different ($p < 0.05$)

As the heat treatment is prolonged, an increase in TOTOX value is observed. Compared to C, the total oxidation recorded in SFO samples supplemented with different concentrations of BBE, and BHT, was significantly reduced ($p<0.05$). Thus, at the end of the heating process, TOTOX value for oil samples supplemented with ZBBE and PBBE decreased in the range of 8-27%. The additive sample with 200 ppm BHT reported a 19% reduction of total oxidation after 12 h exposure to heat treatment. A higher efficiency in inhibiting total oxidation was recorded at BBE applied in an 800 ppm dose compared to BHT. The results obtained after TOTOX determination are in agreement with data recorded in similar studies in which various types of natural extracts were applied in order to limit the total oxidation of sunflower oil subjected to heating processes (Metzner Ungureanu et al., 2020c; Neves, 2020).

Thiobarbituric acid (TBA) assay

The TBA assay is based on the determination of the malondialdehyde (MDA) value in the investigated products. MDA is an essential factor in the production of non-specific odours and flavours. The TBA assay is the most suitable method in determination of oxidative rancidity because MDA is responsible for the specific reaction of thiobarbituric acid (Reitznerová, 2017).

Figure 2 shows the fluctuation of TBA value during high temperature exposure of oil samples supplemented with different concentrations of freeze-dried extracts obtained from blackberry processing by-products and 200 ppm BHT.

TBA indicates the stage of secondary oxidation and the appearance of related products. A high TBA value indicates a decreased oil stability and thermo-oxidation progression. Absorbance decreases with the amount of TBA-MDA complex which implies a higher protection of extracts against secondary oxidation.

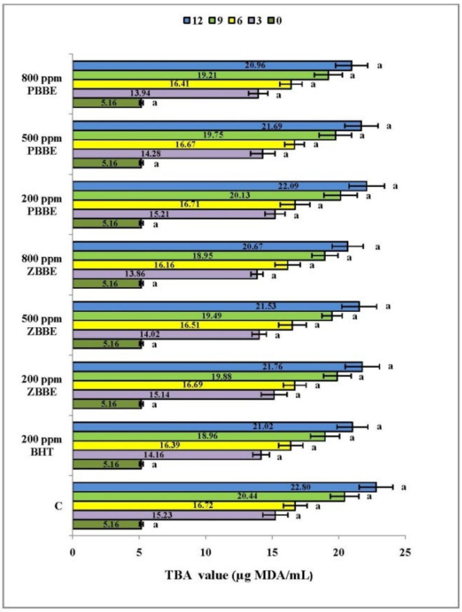

Figure 2. The effect of SFO supplementation with BHT and BBE, on TBA value, during high-temperature heating. One-way ANOVA test was used to compare the means differences registered for each heating period among the oil samples supplemented with BHT and different doses of BBE, relative to C; the values for bars sharing different letters are significantly different ($p<0.05$)

After 3 h of heating at 180ºC, the TBA value was decreased in all investigated samples, as a result of the fact that the rate of destruction of hydroperoxides exceeded their rate of formation. After 6 h of thermal exposure, when the rapport was reversed and the rate of formation of hydroperoxides was exceeded by their rate of decomposition into secondary oxidation products, the TBA value started to increase following this pattern throughout the rest of the heating program. The highest PV value was reported after 3 h of heating when the lowest TBA value was reported, and the lowest PV value was recorded after 12 h of thermal processing when the highest TBA value was noted, which establishes a direct relationship between the increase in hydroperoxide destruction rate and the TBA value. Previous studies conducted on this topic reported the same result regarding the inverse relationship between PV and MDA content (Okhli, 2020). Following the study conducted by Okhli et al. (2020) on the improvement of oxidative stability of sunflower oil by addition of extracts obtained from citron (*Citrus medica* L.) peels, it was found that the decomposition of hydroperoxides and the formation of aldehydes and ketones, contributed to the increase of TBA value simultaneous with the decrease of PV.

At the end of the heating program, ZBBE at the maximum concentration of 800 ppm was the most effective extract against secondary lipid oxidation, leading in a 9% decrease of TBA value compared to C.

CONCLUSIONS

Freeze-dried extracts obtained from blackberry processing by-products showed a strong inhibitory effect against primary and secondary oxidation initiated in sunflower oil following the exposure to 180ºC up to 12 hours. The antioxidant action of freeze-dried extracts obtained from blackberry processing by-products against thermo-oxidation of sunflower oil subjected to intense heat treatment was determined by the presence of polyphenolic compounds.

Supplementation with 200 ppm BHT and various concentrations of freeze-dried extracts obtained from blackberry processing by-products resulted in a significant increase ($p < 0.05$) of the thermo-oxidative stability of sunflower oil subjected to a heating program that simulated the conditions specific to alimentary processing. The effect of freeze-dried extracts ZBBE and PBBE in inhibiting lipid oxidation of sunflower oil, both in early and late stages, was dependent on the dose applied. At a concentration of 200 ppm, BBE showed a lower inhibitory effect compared to the inhibitory action of BHT. At a concentration of 500 ppm, BBE exhibit an inhibitory action similar to that of BHT and at a concentration of 800 ppm, both freeze-dried extracts proved a higher inhibitory effect than 200 ppm BHT.

Following the thiobarbituric acid test, a direct relationship was established between increased hydroperoxide consumption and increased TBA value.

The determination of specific indices for the assessment of oxidative stability revealed a more pronounced inhibitory response in the oil samples supplemented with freeze-dried extract obtained from blackberry processing by-product from an area with a milder climate characterized by higher temperatures and a moderate rainfall regime (ZBBE>PBBE).

The results obtained in this research are of applied importance in the edible oil industry by providing useful information on the evaluation of the ability of freeze-dried extracts obtained from blackberry processing by-products in limiting thermo-oxidative degradation of heat-processed sunflower oil and the evaluation of the opportunity offered by these extracts as potential BHT substitutes.

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STUDY ON THE DIFFERENT LAMBS SLAUGHTERING METHODS AND THE ASSESSMENT OF THE DEGREE OF STRESS THROUGH THE DETERMINATION OF SERUM CORTISOL

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Abstract

All animals reared for meat will experience a level of stress prior to slaughter, which will manifest itself in negative effects on meat quality. The purpose of this research is to find out the level of stress of the animals, at the moment of slaughter, by measuring the cortisol in the serum, as an indirect method of evaluating the quality of the meat. The study was carried out between March and October 2021, on three batches of sheep. Batch 1 was slaughtered conventionally (with stunning), batch 2 was slaughtered traditionally (without stunning) and batch 3 was slaughtered halal (without stunning).). Blood samples were collected from the bleeding wound, and cortisol was dosed in a specialized laboratory by the immunoenzymatic method with chemiluminescence detection. Comparing the analyzed batches, it can be seen that higher average values of the cortisol level were recorded in the batch slaughtered in the halal system, followed by the batch slaughtered in the conventional system and then by the one slaughtered in the traditional system, which obtained the lowest values. The quality of the meat is directly influenced by the way the animals are slaughtered and by the stress during slaughtering.

***Key words**: cortisol, halal, meet, sheep, stress.*

INTRODUCTION

Meat represents the skeletal muscle tissue of mammals, as well as the tissues with which it is in natural adhesions, such as: connective tissue, adipose tissue, bone tissue, nerves, lymph centers, blood vessels, etc. (Wood et al., 2003; Ionescu & Diaconescu, 2010; Petcu, 2013; Predescu et al., 2018).

In recent years, meat has become a controversial topic in public debates, as it involves several dimensions of sustainability. Here, we review trends in global meat consumption, including economic, social, environmental, health and animal welfare aspects (Poore & Nemecek, 2018).

Global meat consumption has increased enormously in recent decades. Notable increases in total consumption were observed in all regions of the world, but especially in Asia, Latin America and Africa. In terms of meat types, pork and poultry currently dominate global consumption and have seen the strongest increases in consumption. The increase in pork consumption is primarily driven by China and several other Southeast Asian countries. In contrast, poultry consumption has sharply increased in all parts of the world, because it is cheaper, often perceived as healthier, and also accepted by various religious precepts, than other types of meat (Mottet & Tempio, 2017). Although to a lesser extent, the global consumption of beef, sheep and goat meat, as well as other types of meat, has also increased over time (Parlasca & Qaim, 2022).

According to structure, pigmentation, chemical composition, meat is classified into (Figure 1):

- white meat, from poultry (chicken, turkey, ostrich etc.) and fish;
- red meat, coming from mammals (cattle, pigs, sheep, goats etc.);
- black meat, from game (Savu & Petcu, 2002; Ionescu & Diaconescu, 2010).

Meat is an important source of energy, and this is due to the high content of minerals (iron, potassium, zinc, phosphorus), but also of vitamins (vitamins from the B complex and vitamins A and D), thus representing a main component in human nutrition (Savu et al.,

2002; Biesalski, 2005; Williamson et al., 2005; Petcu, 2015; Mihai et al., 2021).
Due to its chemical composition, meat is an excellent source of easily digestible proteins, being necessary for a balanced diet (Ionescu & Diaconescu, 2010; Mihai et al., 2021).
Parlasca and Qaim in 2022 mention that muscle tissue (meat) is a rich source of nutrients required for human nutrition, such as protein, vitamins and minerals, so meat consumption can help reduce nutritional deficiencies and promote human health (Headey et al., 2018; Zaharia et al., 2021).

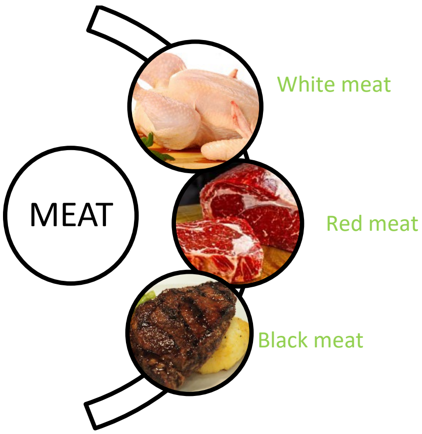

Figure 1. Types of meat available for human consumption

The transformation of animals for slaughter into meat takes place through a chain of events that include handling, loading, transport, unloading, preparation for slaughter and finally slaughter. During all these activities, due to incorrect handling, operational techniques and facilities, the animals are exposed to various stress conditions such as noise, vibration, restriction of movement, lack of food, adverse weather conditions, removal from the group, mixing with unfamiliar animals, overcrowding, increased human contact and inadequate pre-slaughter stunning, which can lead to unnecessary suffering, injury and production losses (Warris, 1995; Chambers et al., 2004; Birhanu, 2020).
Stress is a physiological disorder that is related to mental states associated with threatening or harmful and painful situations (Von Holleben et al., 2010). Stress before slaughter and during the slaughter stages can be differentiated into physical and psychological stress or both types of stress can be manifested (Lawrie, 1966; Mareko, 2005; Grandin, 2007; Adzitey, 2011; Chulayo et al., 2012; Birhanu, 2020; Barrasso, 2021).
Stress is the inevitable consequence of the process of transporting animals from the farm to the slaughterhouse (Ferguson & Warner, 2009; Mekibib et al., 2019).
Such a state of stress can lead to some changes in the concentration of enzymes (creatine-kinase, lactate-dehydrogenase, aspartate-amino-transferase, alanine-amino-transferase, alkaline phosphatase), hormones (catecholamines, cortisol) and other blood constituents , such as glucose and blood cell volume (Knowles & Warriss, 2007), resulting in muscle glycogen depletion, causing a lower rate of post-mortem lactic acid synthesis, high final pH, undesirable meat colour and higher water retention capacity (Chulayo et al., 2012). Furthermore, it causes carcass damage through bruising, hemorrhages, up to total carcass destruction and reduced live weight (Adzitey, 2011). Carcass damage results not only in an economic loss for the meat chain, but also a strong indicator of meat quality (Figure 2) (King et al., 2006; Adzitey, 2011).
The detection of stress in animals at the time of slaughter is necessary to manage the slaughter process in order to produce good quality meat (Ferguson & Warner, 2009; Pérez-Linares et al., 2015; Birhanu, 2020).
The slaughter process is complex, being characterized by a series of stressful stages, caused by many factors, therefore, the purpose of stunning is to render the animals unconscious during bleeding, without causing pain (Önenç & Kaya, 2004; Barrasso, 2021).
However, it is a common practice for Muslims and Jews to slaughter animals by a religious method, practiced without stunning (Önenç & Kaya, 2004; Barrasso, 2021).
Cortisol is a steroid hormone (glucocorticoid) released by the adrenal glands into the blood during stress period, and measuring its concentration in the blood is reliable and widely used as a good indicator of stress (Gupta et al., 2007; Novak et al., 2013).
The release of cortisol during stress also results in an increase in blood glucose through gluconeogenesis to aid in the rapid metabolism of carbohydrates, fats and proteins, nutrients

needed in stressful situations (Mostl & Palme, 2002; Birhanu, 2020).
The determination of cortisol is one of the most used methods for assessing stress in animals, and it can be measured from blood (serum or plasma), saliva, urine, feces, milk and hair (Casal et al., 2017; Mihai et al., 2021).
Hair cortisol analysis is a non-invasive procedure for detecting chronic stress, because cortisol is incorporated and stored inside the hair (Casal et al., 2017).
A recent study followed the cortisol level in sheep hair, harvested from different skin surfaces, following local treatments, such as: excessive brushing, the administration of hyperstimulating substances that increase blood circulation or a synthetic glucocorticoid. In this study, the dosage of cortisol concentration was carried out through immunological tests, an evaluation modality also applied in the present research (Salaberger, 2016).

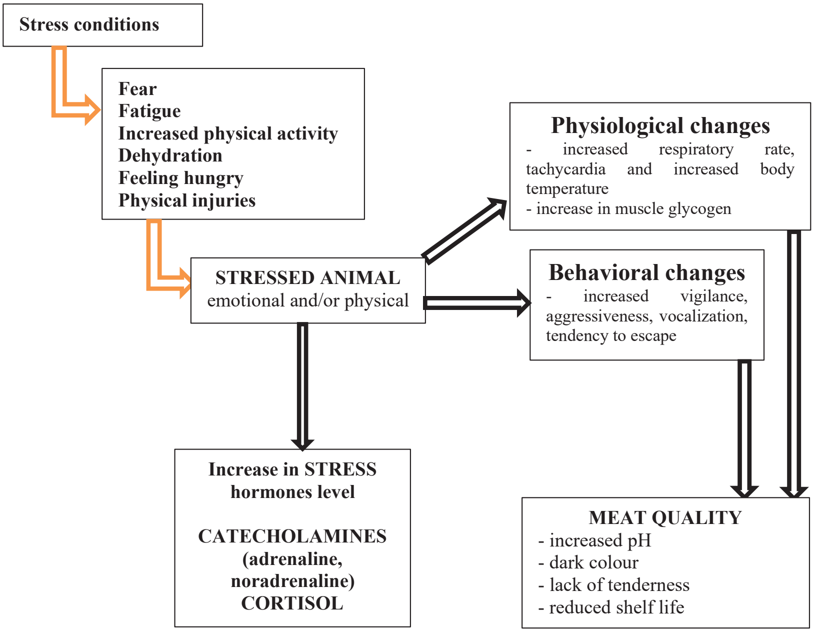

Figure 2. Effects of pre-slaughter stress on animals and meat quality

MATERIALS AND METHODS

The research was carried out between March and October 2021 on 3 batches of sheep, as follows: a batch of conventionally slaughtered sheep (with electric stunning), a batch of traditionally slaughtered sheep (without stunning) and a batch of halal slaughtered sheep (without stun). The following blood samples were collected:
- Batch 1: consisting of 15 blood samples collected from lambs reared on the farm, in March 2021, following conventional slaughter (with electric stunning), in an authorized sanitary-veterinary slaughterhouse.
- Batch 2: consisting of 15 blood samples collected from lambs in April 2021, during Easter, following the traditional slaughter in the households of the population.
- Batch 3: consisting of 15 blood samples collected from a batch of 60 farm-reared lambs of the metis breed, slaughtered in an authorized sanitary-veterinary and halal-certified slaughterhouse, in October 2021, without stunning, by the halal method, specific to Muslims.
In the slaughterhouses, the technological flow of slaughtering sheep was followed and blood samples were collected.
In the case of **conventional slaughter**, the sheep enter the adduction corridor and are electrically stunned, by positioning two electrodes at the head level. Immediately after the stunning, the hanging on the slaughtering

line takes place, and the next stage, namely the bleeding, will take place.

In the case of **halal sheep slaughter**, the animals are slaughtered without stunning, according to religious precepts. Each sheep is contained and the designated person at the slaughterhouse, cuts the blood vessels at the level of the animal's neck in a single movement, using a sharp knife. At the moment of bleeding, *Bismillah* is said.

In the case of **traditional slaughter**, practiced in Romania during the Easter holidays, the lambs are slaughtered without stunning, applying bleeding through the jugular.

The present study aims to carry out laboratory analyzes for the dosage of cortisol from blood samples collected at the time of bleeding (approximately 9 ml of blood collected in a BD Vacutainer - Clot Activator Tube). The blood samples were identified by labeling and transported immediately to a specialized laboratory, cortisol being dosed by the Immunoenzymatic method with chemiluminescence detection.

Cortisol was assayed from serum derived from blood samples (Figure 3) collected from the bleeding wound.

Figure 3. Blood samples collected from the bleeding wound and serum expression mode

For the determination of cortisol, specialized training and laboratory equipment, as well as specific materials and reagents, are required.

RESULTS AND DISCUSSIONS

The determination of the cortisol level in the blood samples collected after the slaughter of the lambs by different slaughtering methods, highlighted different values, and some of them exceed the reference range established by Jackson et al. in 2002, namely 1.40-3.10 μg/dL.

Results and discussions regarding the influence of the method of slaughter on the cortisol level in blood

Stress before slaughter has a negative impact on the hormonal system of animals and implicitly on meat quality (D'Eath et al., 2010; Mihai et al., 2021).

Study 1 - the influence of slaughter method on cortisol levels in blood collected from conventionally slaughtered lambs

Following the analysis of the cortisol level of the 15 blood samples collected from conventionally slaughtered lambs in March 2021, it was observed that 12 of the total samples had higher values compared to the reference range (1.40-3.10 μg/dL), and 3 samples recorded optimal values.

The highest value can be observed in sample number 8 (7.52 μg/dL), being 2.4 times higher than the maximum of the reference interval.

The results obtained after the cortisol dosage from the samples of batch 1 are presented in Table 1.

Table 1. Results obtained following cortisol dosing by immunological examination
in lambs slaughtered in a conventional system from batch 1

No.	Slaughtering date	Sex	Age	Body weight	Cortisol level µg/dL
1.	25.03.2021	M	4 months	10 kg	3.02
2.	25.03.2021	M	4 months	11 kg	**5.25**
3.	25.03.2021	M	4 months	10 kg	**3.74**
4.	25.03.2021	M	4 months	12 kg	**4.93**
5.	25.03.2021	M	4 months	10 kg	2.08
6.	25.03.2021	M	4 months	10 kg	**6.41**
7.	25.03.2021	M	4 months	11 kg	**5.04**
8.	25.03.2021	M	4 months	10 kg	**7.52**
9.	25.03.2021	M	4 months	13 kg	**4.56**
10.	25.03.2021	M	4 months	10 kg	**4.86**
11.	25.03.2021	M	4 months	10 kg	**4.46**
12.	25.03.2021	M	4 months	10,5 kg	**4.89**
13.	25.03.2021	M	4 months	11 kg	2.46
14.	25.03.2021	M	4 months	10 kg	**6.06**
15.	25.03.2021	M	4 months	11.5 kg	**3.95**

Study 2 - the influence of slaughter method on cortisol levels in blood collected from traditionally slaughtered lambs

Following the analysis of the cortisol level of the 15 blood samples collected from traditionally slaughtered lambs in April 2021, it was observed that 2 of the total samples had higher values compared to the reference range (1.40-3.10 µg/dL), and 13 samples recorded optimal values. The average value obtained for all analyzed samples falls within the reference range, being 2.22 µg/dL.
The results obtained after the cortisol dosage from the samples of batch 2 are presented in Table 2.

Table 2. Results obtained following cortisol dosing in lambs slaughtered in the traditional system from batch 2

No.	Slaughtering date	Sex	Age	Body weight	Cortisol level µg/dL
1.	29.04.2021	M	4 months	10 kg	**5.69**
2.	29.04.2021	M	4 months	12 kg	**5.42**
3.	29.04.2021	M	4 months	12 kg	1.57
4.	29.04.2021	M	4 months	14 kg	1.91
5.	29.04.2021	M	4 months	10 kg	1.43
6.	29.04.2021	M	4 months	11 kg	1.48
7.	29.04.2021	M	4 months	12 kg	1.51
8.	29.04.2021	M	4 months	13 kg	1.63
9.	29.04.2021	M	4 months	14 kg	1.41
10.	29.04.2021	M	4 months	11 kg	1.40
11.	29.04.2021	M	4 months	15 kg	1.41
12.	29.04.2021	M	4 months	13 kg	1.42
13.	29.04.2021	M	4 months	15 kg	2.77
14.	29.04.2021	M	4 months	13 kg	2.52
15.	29.04.2021	M	4 months	14 kg	1.77

Study 3 - influence of slaughter method on cortisol levels in blood collected from halal slaughtered lambs

In October 2021, in a specialized laboratory, 15 blood samples collected from lambs slaughtered in the halal system in an authorized sanitary-veterinary slaughterhouse were analyzed by immunological examination. It can be seen that 13 of the total samples recorded values exceeding the reference interval (1.40-3.10 µg/dL), only two samples recording an optimal cortisol level. Sample number 10 recorded the highest value, namely 8.76 µg/dL.
The results obtained after the cortisol dosage from the samples of batch 3 are presented in Table 3.

Table 3. Results obtained after cortisol dosing in lambs slaughtered in the halal system from batch 3

No.	Slaughtering date	Sex	Age	Body weight	Cortisol level μg/dL
1.	20.10.2021	M	4 months	10 kg	**3.89**
2.	20.10.2021	M	4 months	11 kg	**7.25**
3.	20.10.2021	M	4 months	11 kg	2.41
4.	20.10.2021	M	4 months	10 kg	**5.43**
5.	20.10.2021	M	4 months	10 kg	3.08
6.	20.10.2021	M	4 months	10 kg	**6.31**
7.	20.10.2021	M	4 months	10,5 kg	**5.04**
8.	20.10.2021	M	4 months	10 kg	**3.52**
9.	20.10.2021	M	4 months	10 kg	**3.56**
10.	20.10.2021	M	4 months	11,5 kg	**8.76**
11.	20.10.2021	M	4 months	10 kg	**4.18**
12.	20.10.2021	M	4 months	10 kg	**4.25**
13.	20.10.2021	M	4 months	12 kg	**3.25**
14.	20.10.2021	M	4 months	10 kg	**4.55**
15.	20.10.2021	M	4 months	10 kg	**5.05**

Results and discussions regarding the statistical analysis of the data

The results obtained from the summary statistics (mean values, standard error of the mean (SEM), standard deviation, maximum and minimum values) of serum cortisol samples collected from slaughtered animals are presented in Figure 4.

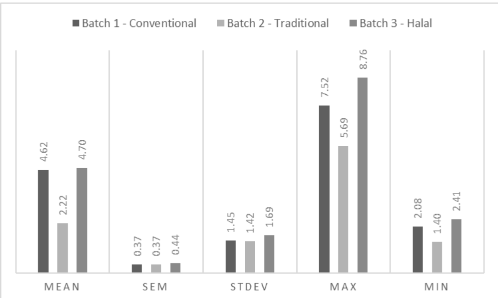

Figure 4. Summary statistics of serum cortisol level in lambs (μg/dL)

Comparing the analyzed batches, it can be seen that higher values of the cortisol level were recorded in the batch slaughtered in the halal system, followed by the conventional slaughtered batch and then by the traditional slaughtered batch.

Batch 1 was slaughtered in March, batch 2 in April and batch 3 in October, months with relatively similar temperatures, ambient temperature being a very important factor in inducing animal stress.

The highest value was recorded in a sample from group 3 (8.76 μg/dL), and the lowest value was recorded in group 2 (1.40 μg/dL).

The results for the three batches, were analyzed statistically by applying ANOVA one-way analysis of variance test, using the GraphPad Prism Statistical Software and the results obtained showed significant differences between the examined batches ($P<0.05$) (Figure 5).

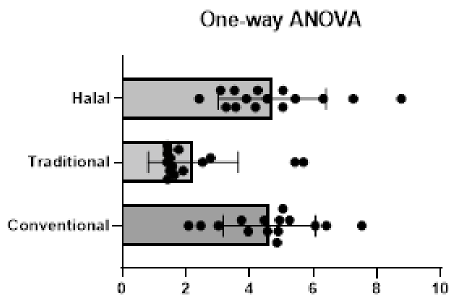

Figure 5. Summary statistics of ANOVA test

In order to determine the significance of the differences between the experimental groups, the t-test (student test) was applied.

The results obtained from the summary statistics of t-test (Student test) for all pairwise comparisons are presented in Table 4.

Table 4. Summary statistics of serum cortisol level in ovine - student test

Batches analyzed	P - value	Significantly different (P<0.05)	Mean ± SEM
Conventional - Traditional	0.0009	Yes	4.62 ± 0.3743, n=15 2.22 ± 0.3654, n=15
Conventional - Halal	0.8533	No	4.62 ± 0.3743, n=15 4.70 ± 0.4373, n=15
Traditional - Halal	0.0004	Yes	2.22 ± 0.3654, n=15 4.70 ± 0.4373, n=15

Batch 1 slaughtered conventionally and batch 2 slaughtered traditionally obtained statistically significant differences (P<0.05) (Figure 6).

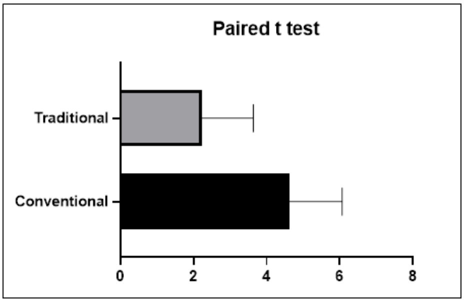

Figure 6. Mean value between batch 1 and batch 2

Batch 1 slaughtered conventionally and batch 3 slaughtered halal obtained statistically insignificant differences (P>0.05) (Figure 7).

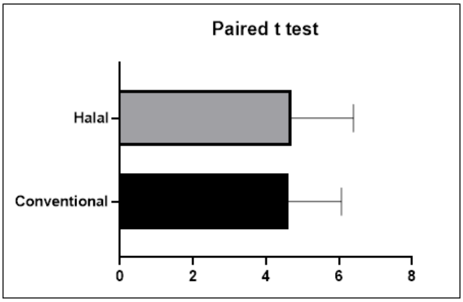

Figure 7. Mean value between batch 1 and batch 3

Batch 2 slaughtered traditionally and batch 3 slaughtered halal obtained statistically significant differences (P<0.05) (Figure 8).

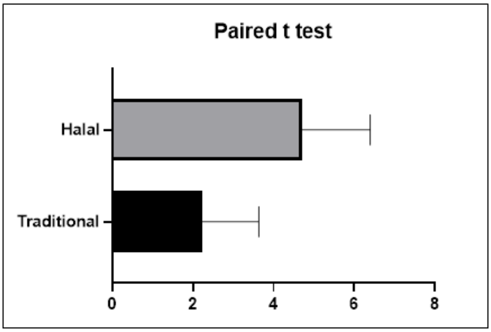

Figure 8. Mean value between batch 2 and batch 3

CONCLUSIONS

In the slaughterhouse in study 1, the technological stages of suppressing the animal's life by the conventional method were observed, and no accidental falls of the lambs were observed in the adduction corridor.

Samples collected from lambs slaughtered in the traditional system in households obtained lower average values (2.22 μg/dL) of the cortisol level, compared to the average values of blood samples collected from lambs slaughtered conventionally (4.61 μg/dL) and halal (4.70 μg/dL), a fact that most probably correlates with the way the animals are reared, with the fact that they do not suffer from transport stress and with the fact that the animals are not kept in crowded groups.

Following the statistical analysis of the three batches, analyzed by the ANOVA one-way test it resulted that there were significant differences within the cortisol levels recorded for all the batches (P<0.05).

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SENSORY AND PHYSICO-CHEMICAL CHARACTERISTICS OF MUFFINS OBTAINED FROM NON-CONVENTIONAL AGLUTENIC FLOURS

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Abstract

In this work we obtained and characterized gluten-free muffins from almond and coconut flour. Three distinct muffin recipes were designed with the two types of flour in different proportions: Var. 1 (100% almond flour), Var. 2 (50:50) almond flour: coconut flour and Var. 3 (80:20) almond flour: coconut flour. The muffins were analyzed from a sensory point of view (using the hedonic method) by a group of 25 consumers, evaluating: external appearance, appearance on the section, taste, smell, aroma, texture, color and consistency. Overall, the highest score was recorded in Var. 3, followed by Var. 1. The taste, color and aroma of the three variants were appreciated very well, but differences could be observed in the appearance on the section and texture. The level of acceptability by consumers was high for all muffin variants obtained. Moisture (34.8 ± 0.7 - 37.5 ± 0.51%), height/diameter ratio (0.4 ± 0.03 - 0.6 ± 0.026), porosity (68.2 ± 2.3 - 72.7 ± 1.8%) and elasticity (48.58 ± 1.4 - 54.9 ± 1.63%), polyphenol content (47.15 ± 2.21-128.4 ± 3.47 mg gallic acid/g) and the antiradical activity were evaluated (RSA: 68.2 ± 3.41 - 63.7 ± 2.6%).

Key words: *almond flour, coconut flour, muffins, physicochemical characteristics, sensorial evaluation.*

INTRODUCTION

Studies according to which gluten affects the digestive (Humbert et al., 2006; Biesiekierski et al., 2011; Herfarth et al., 2014) and neurological (Ford, 2009; Jackson et al., 2012) systems of consumers, the context in which the disease celiac disease is very widespread among humans (Viljamaa et al., 2005; Cosnes et al., 2008; Ford, 2009; Leonard et al. 2017), has led the flour products industry to develop new products by substituting wheat flour with gluten-free flours.

Moreover, the current consumer trend is to change the lifestyle, and implicitly the diet (Petcu C.D. et al., 2019). Thus, there are people who adopt the gluten-free diet, even if they do not have celiac disease, or the ketogenic diet (low carbs) even if they are not diabetic or overweight. In this context, the demand for dietary products is increasing, which is why a particular interest among researchers has been shown in replacing wheat flour with rye, sorghum, coconut, almond, pea, soy, rice, corn, chickpea, quinoa, amaranth (Man et al., 2014; Andersson, 2016; Javaria et al., 2017; Păucean et al., 2017; Stoin et al., 2018; Ghinea et al., 2019; Shatta et al., 2019; Ramya and Anitha, 2020; Roshiya et al., 2022; Hopkin et al., 2022) in obtaining bakery and pastry products.

Nucifera flours are an excellent alternative to wheat flour, with high fat and protein content, respectively lower carbohydrate content (Hopkin et al., 2022).

Due to its chemical composition (proteins: 14-18%, fats: 11-14%, carbohydrates: 60%), coconut flour is also suitable for the ketogenic diet (Hopkin et al., 2022), being very rich in fiber nutrition (double that of wheat bran, and 4 times that of oat bran). Coconut flour also contains globulin and 60 g of dietary fiber (hemicellulose, cellulose and lignin) (Kwon et al., 1996; Trinidad et al., 2006; Yalegama and Chavan, 2006; cited by Jiamjariyatam et al., 2021). According to the study carried out by Arancon (1999), cited by Ramya and Anitha, (2020), coconut flour presents benefits for consumers by contributing to the control of cholesterol and blood sugar levels, as well as to the prevention of colon cancer.

With a fat content of 35-40%, proteins 20-25% and carbohydrates 10-15%, almond flour being gluten-free, is an alternative to wheat flour. At the same time, it also has antioxidant activity conferred by polyphenolic compounds (Takemoto et al., 2001) (Yildiz and Gocmen, 2020).

Due to their wide popularity among consumers, classic muffins and those obtained from gluten-free flours have been the subject of numerous research studies. The total or partial replacement of wheat flour with gluten-free flours has led to very good results. Sensory, physicochemical and nutritional characteristics were studied (Ramya & Anitha, 2020; Stoin et al., 2018; Hopkin et al., 2022; Moss et al., 2022).

Thus, Stoin et al. (2018) reported that by substituting 20, 40 and 40% respectively of wheat flour with almond flour, the acceptability increased directly proportional to the degree of substitution, but the baking parameters (elasticity, porosity, diameter and height) of the muffins from almond flour were inferior to those of the control group. Hopkin et al. (2022) observed increases in the volume of muffins that had a higher content of almond flour compared to those that had a predominantly coconut flour content. Also, almond flour resulted in softer products and better acceptability (Ghinea et al., 2019; Hopkin et al., 2022).

The results of the studies carried out by Ramya & Anitha, (2020) showed that the addition of 25% coconut flour in the dough for muffins improved the sensory and physicochemical characteristics of the obtained samples and, increasing their nutritional value. Similar results were reported by Jiamjariyatam et al. (2021). The use of almond or coconut flour caused a substantial increase in the level of protein in these muffins compared to classic muffins and reduced hardness and fragility in the case of biscuits, but increased crunchiness (Jiamjariyatam et al., 2021). The water content and water absorption capacity of samples from coconut or almond flour is lower than that obtained from wheat flour (Ramya and Anitha, 2020; Stoin et al., 2018).

The polyphenol content of muffins made from almond flour was higher than that of muffins made from wheat flour. An improvement in the antioxidant activity of (Ghinea et al., 2019) was also observed.

Starting, on the one hand, the results reported by Jiamjariyatam et al. (2021), Makinde and Eyitayo, (2019) and Ghinea et al. (2019), who support the fact that bakery products made with coconut flour mixtures present benefits for obese, diabetic, cardiovascular or constipated people, and on the other hand, noting the precariousness of studies related to the use of almond flour in association with the coconut one, we decided to approach the present research in order to evaluate the sensory attributes, mechanical and chemical properties of muffins.

MATERIALS AND METHODS

Obtaining muffins

Three distinct muffin recipes were designed by combining almond flour (AF) with coconut flour in different proportions: Variant 1 (100% almond flour – AF 100), Variant 2 (50:50) almond flour: coconut flour coconut (AF50:CF50) and Variant 3 (80:20) almond flour: coconut flour (AF80:CF20). The ingredients used were: almond flour, coconut flour, eggs, maple syrup, almond milk, sunflower oil, baking powder, vanilla essence, salt.

Beat the eggs and maple syrup for 2-3 minutes until the mixture has doubled in volume. Added the oil, almond milk and vanilla essence. It was mixed again for complete homogenization. Separately sifted: almond flour, salt and baking powder, then mixed a little. This mixture was poured over the previously prepared composition and mixed. After the mixture was homogeneous, it was poured into molds and placed in a preheated oven at 220°C for 13-15 minutes.

Table 1 Recipes for gluten-free muffins

Ingredients	Variant 1 (AF 100)	Variant 2 (AF50:CF50)	Variant 3 (AF80:CF20)
Almond flour, g	250	125	200
Coconut flour, g	-	125	50
Eggs, g	150	150	150
Maple syrup, g	200	200	200
Almond milk, g	70	70	70
Oil, g	100	100	100
Baked powder, g	7	7	7
Vanilla essence, g	0.5	0.5	0.5
Salt, g	3	3	3

Moisture determination

The moisture content of the muffins was determined by calculating the mass loss as a result of heating to 105°C.

Determination of the height/diameter ratio

Based on this report, the degree of development of the muffins taken for analysis can be appreciated. At values of this ratio of 0.4, it is considered that the muffins are well developed, above this value it is considered a superior product, and for lower values, the product is considered inadequate, and flattened. This indicator was determined immediately after cooling the muffins.

Porosity determination

Porosity (the volume of air voids in the product) was determined by measuring the diameter and height of a cylinder cut from the muffins, which was then weighed and calculated with the relation:

$$Porosity\ (\%) = \frac{V - \frac{m}{\rho}}{V} 100 \qquad (1)$$

where:

V - the volume of the core cylinder [cm^3];

m - core cylinder mass [g];

ρ - flour density [g/cm^3],

$\rho_{almond\ flour}$ - 0.5 [g/cm^3],

$\rho_{cocos\ flour}$ - 0.5 [g/cm^3],

Determination of the elasticity of muffins

Muffin springiness was measured by pressing half a muffin for 1 minute and measuring its height at the highest point before and after pressing. Thus, half a muffin is placed on a flat surface and its height is measured. The other half of the muffin placed on the same flat surface is gently pressed with a plunger until it reaches half of the original height and the tension is maintained for 1 minute. The plunger is lifted and the muffin is left for another minute to return to its original shape. Measure the height again at the highest point and use the following relationship to calculate the elasticity:

$$Elasticity\ (\%) = \frac{H\,2}{H\,1} \times 100 \qquad (2)$$

where:

H_1 - the height of the muffin before pressing [mm];

H_2 - the height of the muffin after pressing and its return to the initial position [mm].

Determination of polyphenol content

Total polyphenols content was determined using the Folin-Ciocalteu method (Apak et al., 2008). The results were expressed in mg gallic acid/g (Moldovan et al., 2022).

Determination of free radical scavenging activity

The free radical scavenging activity (RSA) was determined according to the method described by Hue et al., (2020), by the spectrophotometric method with ethanolic DPPH (1,1-diphenyl-2-picrylhydrazyl) 0.1 mM, reading the absorbances of the ethanolic extracts of muffins at 517 nm. For the RSA calculation, the relationship was used:

$$RSA\ (\%) = \frac{A_C - A_S}{A_C} 100 \qquad (3)$$

where:

A_c - absorbance of control;

A_s - absorbance of the sample

Sensory evaluation

The evaluation of the sensory characteristics was carried out after baking the muffins. They were allowed to cool and then the external appearance, appearance on section, taste, smell, aroma, texture, color and consistency were evaluated. These characteristics were evaluated by the hedonic scoring method from 1-5. The sensory examination was carried out by a group of 25 consumers who rated these characteristics on the basis of a sensory sheet. The sensory analysis ensured that the muffins had a specific shape, the presence/absence of possible shape defects (deformations, flattening, crushing, cracks, etc.) was checked. As a color standard for the surface of the muffins, the yellow-copper color was considered, without stains / traces of burns, soot, etc., and in the section golden color. The appearance in the section was evaluated, being properly appreciated the homogeneous, with uniform porosity, well baked. The smell was evaluated immediately after sectioning the muffins, it being characteristic, without foreign smells. Also, the specific taste of muffins was appreciated, pleasant, without unusual tastes (sour, rancid, bitter).

RESULTS AND DISCUSSIONS

The mechano-chemical characteristics of the muffins studied are presented in Table 2 and in Figure 1.

Table 2 Mechano-chemical characteristics of the muffins

Parameter	Variant 1 (AF 100)	Variant 2 (AF50:CF50)	Variant 3 (AF80:CF20)
Moisture, %	34.8±0.7	37.5±0.51	35.2±0.6
H/D ratio	0.6±0.04	0.4±0.05	0.52±0.04
Porosity, %	72.7±1.8	68.2±2.3	71.8±1.9
Elasticity, %	54.9±1.63	48.58±1.4	53.3±1.3

The humidity of the muffins varied between 34.8 ± 0.7 - 37.5 ± 0.51%. Muffins obtained exclusively from almond flour (AF 100) had a lower water content (34.8%) compared to those obtained from a mix (AF50: CF50) of almond flour and coconut flour in equal parts (37.5%). Hence the conclusion that the addition of coconut flour increases the humidity of the muffins. Similar results were reported by Hopkin et al., (2020), respectively Ramya and Anitha, (2020). It was also found that the water retention capacity increased with the increase in the amount of coconut flour in the dough (Ramya and Anitha, 2020). In contradiction to these results, Makinde & Adeyemi (2018) found higher moisture content in CF biscuits than in AF ones. In the case of partial substitution of rice flour with almond flour, a better water retention tendency was found, directly proportional to the degree of substitution (Stoin et al., 2018).

The best value of the height / diameter ratio was recorded for version 1 (muffins from AF 100) - 0.6, while for version 3 (AF80:CF20) the value of 0.4 was calculated, and for version 2 (AF80: CF20) - 0.52. Even with the 50% substitution of almond flour with coconut flour, the muffins developed well. The lower degree of substitution of AF with CF ranked the muffins obtained in the higher category.

The best porosity was recorded in Variant 1 (AF100) - 72.7 ± 1.8%, and the lowest in variant 2 (AF50: CF50) - 68.2 ± 2.3%. The addition of CF leads to an increase in the content of dietary fiber (Poonam and Tech, 2013, cited by Jiamjariyatam et al., 2021), and as a result, the density of the product increases, that is, the porosity is reduced. The presence of CF in muffins determined the reduction of their volume (Ramya & Anitha, 2020). These results also correlated with those reported by Stoin et al. (2018), who found in almond flour muffins porosity of 66.9%, but the increase in FA content (and the reduction of that of rice) was inversely correlated with porosity. The results of the study carried out by Gillespie & Ahlborn (2021) showed that the volume of bread obtained from almond flour was superior to other types of bread obtained from mixes with oat flour.

The experimental results show that the elasticity of the muffins was influenced by the ratio between AF and CF. By comparison with muffins obtained exclusively from AF, those with CF presented less elasticity. Thus, muffins with 100% AF had the highest elasticity (54.9%). The increase in the degree of substitution of FA varied inversely proportional to the elasticity of the muffins (Stoin et al., 2018). The decrease in the elasticity of muffins with CF was also reported by Hopkin et al., (2020).

The content of polyphenols and RSA of the muffins is presented in Figure 1. Muffins with exclusive AF had the highest content of polyphenols (128.4 mg gallic acid/g), while in version 2 of muffins it was substantially reduced (47.15 mg gallic acid/g). This fact is explained by the high content of polyphenols (625 mg/100 g) present in almond flour (Siqueira et al., 2015). The substitution of FA with CF determined the reduction of the content of polyphenols in direct correlation with the degree of substitution. Ghinea et al. (2019) reported that the substitution of wheat flour with almond flour led to increases in the total content of polyphenols in muffins.

Radical scavenging capacity was directly correlated with higher AF content. Thus, the highest RSA value (68.2%) was observed in muffins with AF 100, while the lowest value (63.7%) was observed in muffins with the highest CF content. Considering that the value of 89.74 ± 0.8% for the DPPH radical scavenging activity found by (Naseer et al., 2021), for the almond cake, indicates its superior bioactive properties, it can be stated that the muffins from the present study have good antioxidant activity. Ghinea et al. (2019) observed that replacing wheat flour with FA resulted in an increase in antioxidant activity.

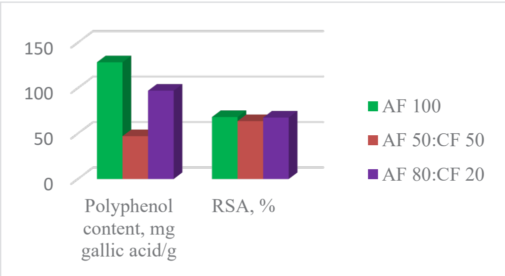

Figure 1. Polyphenol content and RSA

Following the evaluation of the sensory characteristics, the average of the recorded values is presented in Figures 2-4. In variant 1 (AF 100) of muffins, the taste, smell, aroma and color were appreciated very well, but the external appearance, the one on the section, the consistency and the texture were depreciated.
Muffin variant 2 (AF: CF - 50: 50) received high scores for colour, consistency, aroma, texture and taste, but was penalized for appearance and section. In the case of variant 3 (AF: CF - 80: 20), high scores were recorded for: colour, consistency, smell, aroma and texture. And in the case of variant 3, the appearance has been depunctated.

Figure 2. Muffins with AF and mix of AF with CF

According to the average of the assessed sensory characteristics, the highest score was recorded for variant 1, closely followed by variant 3.

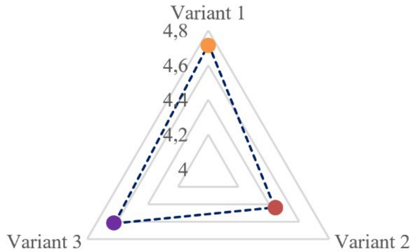

Figure 3. The mean of the sensory acceptability characteristics of the three types of muffins

If the taste, color and aroma of the three variants were appreciated close to the maximum, differences could be observed in the appearance per section and texture. Muffins made entirely from almond flour scored the highest for appearance per section, and those with equal mixes of almond flour and coconut scored the lowest.
In the specialized literature, Wilderjans et al. (2013) argue that the appearance of muffins obtained from almond flour is less appreciated once the proportion of almond flour increases, but Stoin et al. (2018) argue otherwise.
The texture of the muffins was improved by increasing the proportion of almond flour. This fact was correlated with the data from the specialized literature (Hopkin et al., 2022, cited by Moss et al., 2022).

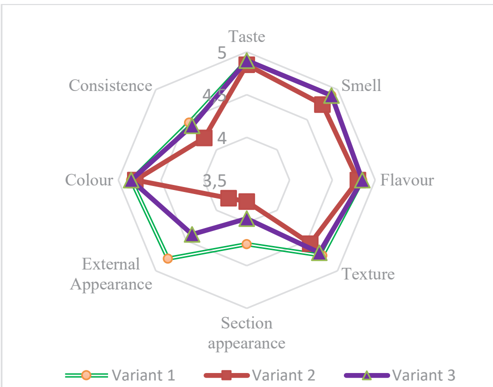

Figure 4. Sensory characteristics of the three types of muffins

The results of the study carried out by Stoin et al. (2018) show that the texture of muffins was detuned with increasing almond flour content. The aroma and taste of the muffins were appreciated with very high scores. The taste and aroma conferred by almond flour, similar to that of walnut, was also appreciated in other specialist studies (Wilderjans et al., 2013).

CONCLUSIONS

The topic addressed led to the formulation of several conclusions.
The level of acceptability by consumers was high for all the variants of muffins obtained, the variants with a higher content of almond flour being rated better.
The substitution of almond flour with coconut flour led to an increase in the water content of the finished products.
Porosity and elasticity were influenced by the ratio of AF and CF.
Antioxidant activity (RSA) and polyphenol content was directly influenced by almond flour content.
The study is worth continuing to highlight other aspects regarding the optimal use of non-conventional flours in the bakery, pastry and confectionery industry.

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EFFECT OF PRE-SLAUGHTER WEIGHT ON CARCASS QUALITY IN PIGS OF IRISH ORIGIN

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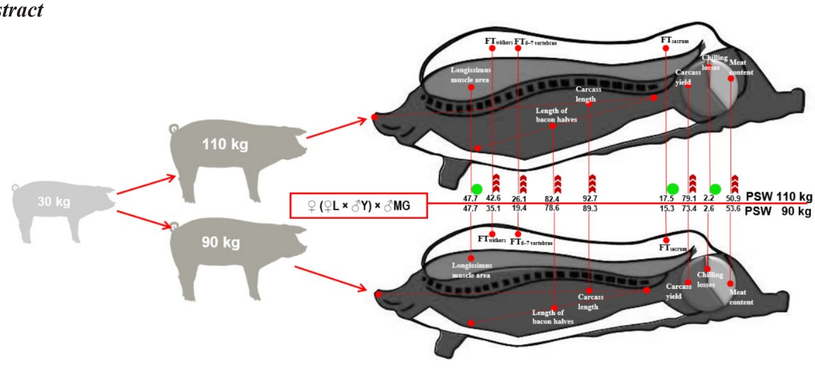
Abstract

To determine the effect of pre-slaughter weight on carcass performance of pigs of Irish origin, two groups of 14 pigs were formed with live weights of 90 and 110 kg per group, including 7 barrows and 7 gilts. After slaughter and carcass fabrication, carcass indicators were measured and the relationship to pre-slaughter weight was examined. For 110 kg pigs, a 1 kg inrease in pre-slaughter weight increased carcass chilling losses by 0.1% ($r = 0.41$; $p<0.001$), decreased carcass yield by 0.81 kg ($r = -0.5$; $p<0.001$), increased carcass length by 0.48 cm ($r = -0.64$; $p<0.001$), increased fat thickness over withers by 0.76 mm ($r = 0.38$; $p<0.001$), increased fat thickness over 6th-7th thoracic vertebrae by 0.76 mm ($r = 0.37$; $p<0.001$). For 90 kg pigs, an 1 kg increase in pre-slaughter weight increased fat thickness over 6th-7th thoracic vertebrae by 0.45 mm ($r = 0.45$; $p<0.001$), increased fat thickness in sacrum by 0.89 mm ($r = 0.16$; $p<0.001$), and decreased meat content by 0.89% ($r = -0.28$; $p<0.001$). An increase in pre-slaughter weight did not lead to a decrease in the carcass quality class.

Graphic abstract



Key words: *bacon halves, carcass yield, carcass length, fat thickness, meat content.*

INTRODUCTION

Breeding for meat production has been a major goal in pig farming for decades (Tribout et al., 2010). The composition and quality of the carcass is contingent on the pre-slaughter weight, which is due to the intensity of growth and influence of consumer or market demand. Balancing the performance of pigs and consumer demand in the pork market increases the profitability of pig farms (Yang et al., 2012). Indicators of slaughter quality of pigs in different ways affect the further evaluation of meat cuts, which are evaluated differently by consumers in different regions. In addition, slaughter quality indicators are closely interrelated, including carcass yield, lean meat content, and fat thickness, which are the focus for both pork producers and consumers (Lebret et al., 2014). It has long been known that when a pig's body reaches a state of puberty, exchangeable energy, nutrients and minerals of fodder cease to be expended on the formation and enlargement of skeletal muscles and bones. The energy is then converted into fat, which is first deposited under the skin, then deposited between the muscles, and then between the bundles of muscle fibers (De Smet et al., 2004). Based on this pattern, genetic companies and pork producers have focused their research on breeding heavier pigs to provide lean pork while maintaining acceptable levels of fat (Price et al., 2019). Pre-slaughter or market weights vary around the world with pigs slaughtered at live weight 115 kg in South Korea, 160 kg in Italy, 100-140 kg in the United States and 110-120 kg in most European and Asian countries (USDA, 2022). Thus, the pig industry is now focused on increasing the pre-slaughter weight, which is likely to continue in the search for the optimal ratio between weight and carcass quality in the near future (Hwang et al., 2020; Kim et al., 2005; Wu et al., 2017).

The influence of the slaughter weight of pigs on the quality of their carcasses, both before and now, is devoted to a lot of research. Thus, the evaluation of pork eating quality attributes found the highest consumer taste and juiciness ratings for pork from pigs with greater loin fat thickness. And for the general acceptability of pork the minimum fat thickness of 21–30 mm is required (Hoa et al., 2021). There is a linear positive relationship fat thicknesses in different parts of the carcass with pre-slaughter weight (Choi et al., 2019; Virgili et al. 2003). These past studies found that fat thickness increased by 0.18 mm per 1 kg increase in the pre-slaughter weight of pigs from 85 to 150 kg. This change in fat thickness was due to growth in pre-slaughter weight by 4.67%, and other changes in fat thickness depended on other factors (Park et al., 2013). Growth intensity was negatively correlated to fat thickness over withers was significant and negative up to 20 weeks, and after 20 weeks of fattening it changed to positive and lost statistical significance before slaughter (Park et al., 2018). The researchers also reported that 37.81% of the variability in fat thickness over 6^{th}–7^{th} thoracic vertebrae was due to the influence of the pre-slaughter weight factor (Harsh et al., 2017).

Indicators of meat content and its dependence on pre-slaughter weight are important for the whole process of pork production. Researchers have found conflicting data that increasing pre-slaughter weight can lead to both an increase in meat content and a decrease in meat content, depending on the breed of pig (Birta et al., 2020). Increasing the meat content of pig carcasses through targeted feeding is widely used in the practice of pig farming (Hambrecht, 2004). Positive linear relationships have been found in lean meat content with slight increases in pre-slaughter weight in the range from 85 to 120 kg, and at heavier pre-slaughter weights up to 135 kg. Korean researchers also reported that the meat content in the carcass increased by 0.37% for every 10 kg increase in pre-slaughter weight from 88.6 to 122.5 kg (Jeong et al., 2010). Similar findings (Park, 2011) can be observed in other publications, which say that the pre-slaughter weight can be increased from 110 to 125 and 135 kg, respectively, as the meat content does not decrease with increasing pre-slaughter weight. With such an increase in pre-slaughter weight, it is also predicted that the fat thickness will be approximately 24 mm over 6^{th}-7^{th} ribs without compromising the quality and content of meat. Similar reports indicate that despite an increase in pre-slaughter weight from 110 to 130 kg (Kim et al., 2005) and from 125.6 to 152.5 kg (Maiorano et al., 2007) and on a corresponding increase in fat content and fat thickness, lean meat content also increased. It was reported that the content of meat is contingent not only on the ratio of fat and lean tissues, but also depends on the carcass yield. The meat content increases by 0.84 kg, with the increase in carcass yield by 1.0% (Mikhailov, 2011).

However, there are many studies that point to the inexpediency of raising pigs to heavy weights, as this leads to an increase in fat content, and reduced content and quality for pork (Peinado et al., 2011). As the pre-slaughter weight increased from 126 to 168 kg, the carcass yield and fat thickness increased linearly while percentage lean meat decreased (Malgwi et al., 2022). Although feeding to heavier pre-slaughter weights increased the absolute amount of meat on carcass, pork quality deteriorated (Bertol et al., 2015). Other studies (Barducci et al., 2020) havefound weak relationships between pre-slaughter weight and longissimus muscle depth or lean meat yield and a weak relationship between fat thickness and

Longissimus muscle area such that the amount of lean meat does not decrease with heavier pre-slaughter weights. This showed that with increasing weight, pigs lost little of their lean meat. Our hypothesis is to avenge the increase in pre-slaughter weight from 90 kg to 110 kg in pigs of the Irish breeding, finishing in the minds of the industrial pig farm, not to reduce the class of their carcasses. Thus, the aim of this study was to determine the effects of pre-slaughter weight on carcass quality and the nature of the relationship amongst carcass quality attributes in pig carcasses from commercial genotypes of Irish origin.

MATERIALS AND METHODS

The pigs for this study were sourced from 400 commercial hybrids of Irish origin from the genetic firm Hermitage. These pigs were produced from Irish Landrace and Yorkshire F_1 sows, that were bred to semen from the Max Gro synthetic terminal line boars. Pigs were raised and fattened in equal numbers of barrows and gilts in Globinsky Pig Complex (Poltava region, Ukraine).

At an average live weight of 100 kg, all experimental pigs were individually weighed and divided into two groups with an average weight of 90 and 110 kg. From each weight group, 14 pigs were selected for slaughter 7 gilts and 7 barrows for the 90 kg pre-slaughter weight group and 7 gilts and 7 barrows for the 110 kg pre-slaughter weight group. Pre-slaughter pigs from both groups were marked on the waist on each side with numbers using a tattoo hammer to identify carcasses.

All pigs were produced and fed under identical conditions. During the study, pigs were finished in 40 m^2 area pens with 50 pigs in each pen. The material of the buildings and floors, their planning and technological equipment for feeding, watering and maintenance zoohygienic conditions for keeping pigs were identical. Pigs of both sexes were kept together in a pen.

Complete feeding rations (Table 1) with a distribution frequency of 10 times a day were used for feeding. Compound feeds were manufactured and balanced with nutrients and energy in Weda feed production equipment (Dammann & Westerkamp GmbH, Austria) at Globinsky Pig Complex (Poltava region, Ukraine).

Table 1. The structure of the feed ration

Ingredient	Value
Maize grain kibbled (IFN 4-02-866), %	19.0
Wheat grain (IFN 4-05-211), %	35.9
Soybean seeds meal solvent extracted (IFN 5-04-604), %	13.3
Sunflower seeds meal mechanical extracted (IFN 5-27-477), %	8.8
Sorghum grain (IFN 4-04-383), %	12.0
Wheat bran (IFN 4-05-190), %	1.0
Cereals, screenings (IFN 4-02-156), %	10.0

The nutritional value of the diet was sufficient and included the necessary vitamins and micro- and macro-elements necessary for normal fattening pigs (Table 2). Pigs were fed with this diet from their live weight of 60 to 110 kg. The ratio of dry food to water was 1: 3.

Table 2. Nutritional value of feed for pigs fattening

Indicator	Value
Protein content, %	18.0
Lysine content, %	1.0
Oil content, %	2.8
Fiber content, %	4.3
Calcium content, %	0.63
Assimilable phosphorus content, %	0.29
Total phosphorus content, %	0.57
Vitamin A content, IU/kg	10000
Vitamin D content, IU/kg	2000
Vitamin E content, IU/kg	100
Biotin content, μg/kg	100
Assimilable energy, MJ/kg	13.65

All experimental pigs were sent for slaughter to the slaughterhouse of Globinsky Meat Processing Plant (Poltava region, Ukraine). The pigs were held for 24 hours without access to feed and then stunned in the gas chamber Schaller Butina DK 4300 (Butina ApS, Denmark) with death by carbon dioxide asphyxiation. The carcasses were then processed according to generally accepted methods (ISO 3100-1). The following carcass parameters were measured on the hot carcass: carcass length, length of bacon halves, fat thickness at three carcass points (over the withers, over the sacrum, at the level of 6^{th}–7^{th} thoracic vertebrae). After pre-keeping the carcasses at a temperature of 14°C for 1 hour 45 minutes, the carcasses were chilled at a temperature of 4°C for 24 hours. Twenty-four hours after slaughter, the carcasses were fabricated in the meat processing department of Globinsky Meat Processing Plant to determine their characteristics according to existing methods (ISO 3100-1): *Longissimus* muscle area, carcass yield, meat content, cooling losses after 24 hours, weight of chilled carcass.

Warm carcass weight was measured as unchilled carcass weight after slaughter and removal of the head, skin, intestinal tract and internal organs.

Carcass yield was calculated as the percentage of carcass weight, head, legs and visceral fat to the live weight of the pig before slaughter.

Weight of chilled carcass was measured as carcass weight without head, tail, internal fat, genitalia, internal organs and their contents after 24 hours of cooling.

Cooling losses were calculated as the difference between warm carcass weight and chilled carcass weight.

Fat thickness was measured with a millimeter ruler at warm half-carcass in a hanging vertical position including the thickness of the skin.

Carcass length was measured in centimeters with a ruler in a hanging vertical position, along the middle of the cut from the anterior edge of the pubic bone to the front surface of the first cervical vertebra.

The length of the bacon half was measured with a centimeter ruler, in the hanging vertical position of the carcass, along the middle of the cut from the anterior edge of the pubic bone to the middle of the first rib.

The Longissimus muscle area was measured on a transverse section between the last thoracic (or 12^{th}) and first lumbar vertebrae (or 13^{th}). The contour of the cut of the longest muscle of the back was transferred to a transparent film and scanned from the film to an electronic medium using a scanner. Next, we used ImageJ 1.53e software capabilities. The downloaded cut image was calibrated for size using a photo millimeter scale ruler and converted to 8-bit expansion. Next, using the shape selection function and the area analysis function, we measured the area of the cut image. Cut image area measured using this software corresponds to the Longissimus muscle area.

Meat content was calculated as the ratio of the total mass of striated red muscle to the mass of the carcass without the head and forelimbs.

The class of the carcasses was determined by a staff certified classifier in the slaughterhouse after 15 minutes they were fabricated by using the method of calculating the meat content. Each carcass was assigned the appropriate class and marking according to SEUROP (Commission Regulation (EU) 2008). Classes of pig carcasses depended on the content of meat content: S - 60% or more, E - 55% or more, U - 50% or more, but less than 55%, R - 45% or more, but less than 50%, O - 40% or more, but less than 45%, P - less than 40%.

Statistical analysis included: calculation of mean value, standard deviation, errors of standard value, correlation coefficients, coefficient of determination (square of correlation coefficient) and method of constructing a two-dimensional linear mathematical model. The significance of the discrepancy ($p<0.001$) between the slaughter qualities indicators ($n = 28$) was analyzed using Student's t-test. The assessment of the strength of the correlation relationship was determined by the value of its coefficient (r): $0.1<r<0.3$ - weak correlation, $0.3<r<0.5$ - moderate correlation, $0.5<r<0.7$ - noticeable correlation, $0.7<r<0.9$ - high correlation, $0.9<r<1$ - very high correlation. To obtain graphs and perform statistical calculations were used Microsoft Office Excel 2010.

The protocol of the experiment was agreed and approved by the Bioethical Commissions of Animal Care and Use during scientific (experimental) research of Sumy National Agrarian University (ethical approval number BT-22-0122-03). Experimental pigs were used strictly accordingly to Guide for the Care and Use of Laboratory Animals, with Law of Ukraine On protection of animals against inhumane treatment and in accordance with the requirements of Council Directive 86/609/EEC.

RESULTS AND DISCUSSIONS

Increasing pre-slaughter weight from 90 to 110 kg increased ($p<0.001$) warm carcass weight by 21.1 kg or 32.25%, carcass yield by 5.7%, weight of chilled carcass by 20.9 kg or 34.83%, fat thickness over 6^{th}-7^{th} thoracic vertebrae by 6.7 mm or 34.54%, carcass length by 3.4 cm or 3.81%, length of the bacon half 3.8 cm or 4.83% (Table 3).

Table 3. Slaughter rates of pigs with different pre-slaughter weight (n = 28)

Indicators	I Group (90 kg)	II Group (110 kg)	p-value
Warm carcass weight, kg	61.6±0.34[a]	82.7±0.24[b]	<0.001
Carcass yield, %	73.4±0.83[a]	79.1±0.80[b]	<0.001
Weight of chilled carcass, kg	60.0±0.32[a]	80.9±0.64[b]	<0.001
Chilling losses after 24 h, kg	1.6±0.15[a]	1.8±0.43a	0.3257
Chilling losses after 24 h, %	2.6±0.22[a]	2.2±0.47[a]	0.2041
Fat thickness:			
over 6th-7th thoracic vertebrae, mm	19.4±0.80[a]	26.1±0.61[b]	<0.001
over sacrum, mm	15.3±1.24[a]	17.5±0.6[a]	0.0747
over withers, mm	35.1±0.83[a]	42.6±1.08[b]	<0.001
Carcass length, cm	89.3±0.57[a]	92.7±0.41[b]	<0.001
Length of bacon halves, cm	78.6±0.34[a]	82.4±0.47[b]	<0.001
Longissimus muscle area, cm^2	47.7±1.07[a]	47.7±1.07[a]	0.4895
Meat content, %	53.6±0.47[a]	50.9±0.44[b]	<0.001
Carcass class (average)	U	U	

Note: [ab]Means in the same row without common letter are different at p<0.05.

Pigs with a pre-slaughter weight of 90 kg had a higher meat content by 2.7% (p<0.001) than a 110 kg pig. There was no significant difference between the carcasses, the thickness of the fat in the sacrum, and the *Longissimus* muscle area between the carcasses of the two groups of pigs. There was no significant difference between cooling losses after 24 hours, fat thickness over sacrum, and *Longissimus* muscle area in the carcasses of the two groups of pigs.

The correlation between pre-slaughter weight in 90 kg pigs with fat thickness over 6th-7th thoracic vertebrae was direct, moderate and reliable (r = 0.45; p<0.001), with fat thickness over sacrum was direct, weak and also reliable (r = 0.16; p<0.001), with meat content was inverse, weak and probable (r = -0.28; p<0.001). Evaluation of the correlation between slaughter indicators and pre-slaughter weight showed the presence of both direct and inverse interdependence between them (Table 4).

In particular, the correlation between the indicators of cooling losses after 24 hours (r = -0.07; p<0.001), the carcass length (r = -0.18; p<0.001) and the *Longissimus* muscle area (r = -0.24; p<0.001) in pigs with pre-slaughter weight 90 kg had a weak and inverse. Correlation between the carcass yield (r = 0.12; p<0.001), the length of the bacon half (r = -0.06; p<0.001), the fat thickness at the withers (r = 0.16; p<0.001) and pre-slaughter weight in light weight pigs was direct but weak.

Table 4. Correlation between pre-slaughter weight and slaughter indicators (n = 28)

Indicator	Group I (90 kg)		Group II (110 kg)	
	r	p-value	r	p-value
Cooling losses after 24 hours	-0.07	<0.001	0.41	<0.001
Carcass yield	0.12	<0.001	-0.55	<0.001
Carcass length	-0.18	<0.001	0.64	<0.001
Length of bacon halves	0.06	<0.001	0.29	<0.001
Fat thickness over withers	0.16	<0.001	0.38	<0.001
Fat thickness over 6th-7th thoracic vertebrae	0.45	<0.001	0.37	<0.001
Fat thickness over sacrum	0.16	<0.001	0.13	<0.001
Longissimus muscle area	-0.24	<0.001	-0.01	<0.001
Meat content	-0.28	<0.001	0.03	<0.001

The correlation relationship of pre-slaughter weight in 110 kg pigs with the indicator of cooling losses after 24 hours was direct and moderate (r = 0.41; p<0.001), with the indicator of carcass yield was inverse and noticeable (r = -0.55; p<0.001), with the carcass length was straight and moderate (r = 0.64; p<0.001), with the length of the bacon half was straight and weak (r = 0.29; p<0.001), with the fat thickness over withers was straight and moderate (r = 0.38; p<0.001), with the fat thickness over 6th-7th thoracic vertebrae was straight and moderate (r = 0.37; p<0.001).

The correlation between the pre-slaughter weight in heavy weight pigs (110 kg) and the fat thickness over sacrum, the *Longissimus* muscle area and meat yield was statistically insignificant.

It was found that with increasing pre-slaughter weight in 110 kg pigs per 1 kg cooling losses after 24 hours increased by 0.1% (Figure 1 a, b). Moreover, changes in the rate of cooling losses after 24 hours were formed by the impact of pre-slaughter weight by 1.73% and by unaccounted factors by 98.27%. When changing the pre-slaughter weight of 90 kg of pigs, there was no significant increase or decrease in cooling losses after 24 hours.

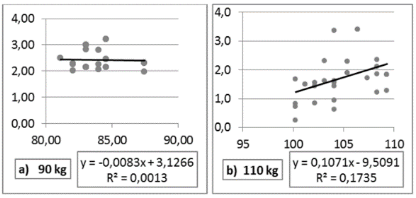


Figure 1. Linear approximation of the dependence of cooling losses after 24 hours on pre-slaughter weight

Fluctuations in pre-slaughter weight in 90 kg pigs did not significantly affect changes in carcass yield (Figure 2 a). With an increase in pre-slaughter weight in 110 kg pigs per 1 kg, carcass yield decreased by 0.81 kg. The detected change in carcass yield was due to the influence of pre-slaughter weight by 3.01% (Figure 2 b).

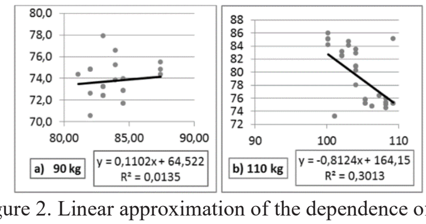


Figure 2. Linear approximation of the dependence of the carcass yield on pre-slaughter weight

No reliable relationship between carcass length and pre-slaughter weight in 90 kg pigs was found (Figure 3 a). The increase in pre-slaughter weight in 110 kg pigs per 1 kg caused an increase in the carcass length by 0.48 cm. Moreover, this dependence of the carcass length was formed under the influence of the pre-slaughter weight by 4.09% (Figure 3 b).

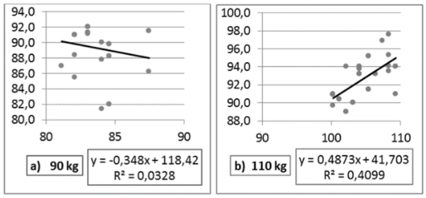


Figure 3. Linear approximation of the dependence of the carcass length on the pre-slaughter weight

Any changes in the pre-slaughter weight in 90 kg pigs did not lead to probable changes in the length of the bacon half (Figure 4 a). Changes in the pre-slaughter weight per 1 kg in the group of 110 kg pigs significantly changed the length of the bacon half of their carcass by 0.24 cm with a force of 0.81% (Figure 4 b).

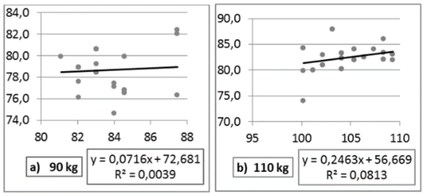


Figure 4. Linear approximation of the dependence of the length of the bacon half on the pre-slaughter weight

Fluctuations in the fat thickness over withers in 90 kg pigs did not depend on the effect of pre-slaughter weight (Figure 5 a). With the increase in pre-slaughter weight in 110 kg pigs per 1 kg, the fat thickness over withers increased by 0.76 mm under the influence main factor by 1.44% (Figure 5 b).

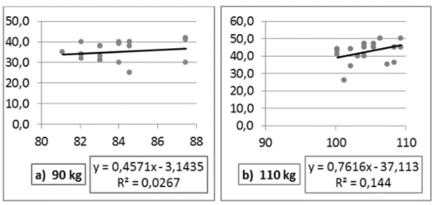


Figure 5. Linear approximation of the dependence of the fat thickness over withers on the pre-slaughter weight

The fat thickness over 6^{th}-7^{th} thoracic vertebrae increased in 90 kg pigs by 1.20 mm, and in 110 kg pigs by 0.42 mm in parallel with the increase in pre-slaughter weight by 1 kg (Figure 6 a, b). Moreover, the main factor in the group of light weight pigs caused a change in the dependent indicator by 2.0% and in the group of heavy weight pigs by 1.4%.

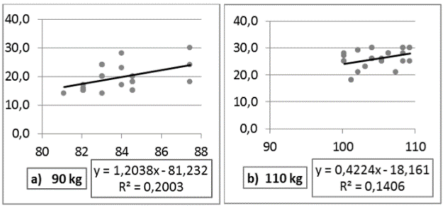


Figure 6. Linear approximation of the dependence of the fat thickness over 6th-7th thoracic vertebrae vertebrae on the pre-slaughter weight

Changes in the pre-slaughter weight in 90 kg pigs led to changes in the fat thickness over sacrum by 0.89 mm with a force of 0.79%. No correlation was found between the fluctuations in the fat thickness over sacrum and the pre-slaughter weight in 110 kg pigs (Figure 7 a, b).

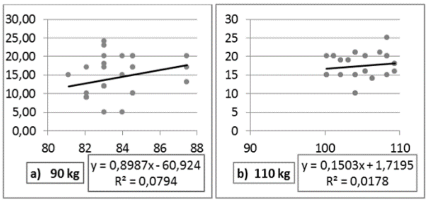


Figure 7. Linear approximation of the dependence of the fat thickness over sacrum on the pre-slaughter weight

The nature of the dependence of meat content and pre-slaughter weight in 90 kg pigs was determined by the influence of the factor trait with a force of 0.76%. This led to a decrease in meat content by 0.89% with growth in pre-slaughter weight by 1 kg. In 110 kg pigs, the meat content did not depend on changes in the pre-slaughter weight (Figure 8 a, b).

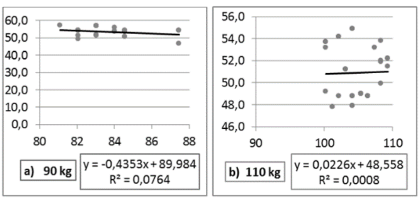


Figure 8. Linear approximation of the dependence of meat content on pre-slaughter weight

The Longissimus muscle area only tended to decrease by 0.87 cm^2 with an growth in pre-slaughter weight by 1 kg in 90 kg pigs and by a decrease of 0.01 cm^2 in 110 kg pigs (Figure 9 a, b).

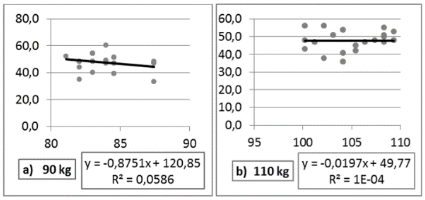


Figure 9. Linear approximation of the dependence of the *Longissimus* muscle area on the pre-slaughter weight

The results of our studies only partially coincided with the report (Makaukii, 2000), which indicated that increasing the pre-slaughter weight of pigs from 60 to 90 kg significantly increased the *Longissimus* muscle area, fat thickness, carcass length and carcass yield. Contrary to the report (Makaukii, 2000), in our study with increasing pre-slaughter weight to 90 kg, the *Longissimus* muscle area and the carcass length tended to decrease, and the carcass yield tended to increase. We found that when the pre-slaughter weight increased by 1 kg, only the fat thickness significantly increased at only two points of the carcass: over 6th–7th thoracic vertebrae by 0.45 mm and over sacrum by 0.89 mm, what is similar to the results where fat thickness increased by 0.18 mm per 1 kg increase in pre-slaughter weight of pigs from 85 to 150 kg (Park et al., 2013).
Also, our findings partially coincided with the published data (Price et al., 2019), which reported that with increasing pre-slaughter weight increased: the *Longissimus* muscle area ($r = 0.24$; $p<0.001$), fat thickness ($r = 0.13$; $p<0.001$). We found similar results with respect to the increase in fat thickness with increasing pre-slaughter weight in both 90 kg pigs and 110 kg analogues. But in light weight pigs the increase in fat thickness over 6th-7th thoracic vertebrae was in combination with growth fat thickness over sacrum, and in heavy weight pigs the growth of fat thickness over 6th-7th thoracic vertebrae was in combination with growth of fat thickness over withers. This result is confirmed by data, which indicate a statistically appreciable effect of pre-slaughter weight on the fat thickness over 6th-7th thoracic vertebrae with a force of 22.09% (Andrieieva, 2020) and 37.81% (Harsh et al., 2017). But in our results the factor of pre-slaughter weight on the fat

thickness over 6^{th}-7^{th} thoracic vertebrae affected much weaker: in 90 kg pigs at 2.00% and in 110 kg pigs at 1.40%. We concluded that fat thickness increased with increasing pre-slaughter weight in pigs, which coincides with reports that fat thickness was greater ($p<0.05$) in heavy pigs with pre-slaughter weight 135 kg (31.2 mm) and 125 kg (29.3 mm), compared with light 115 kg analogues (25.0 mm) (Oh et al., 2022). We obtained a direct linear relationship between pre-slaughter weight and fat thickness similar to other published results (Choi et al., 2019; Malgwi et al., 2022; Virgili et al., 2003).

The *Longissimus* muscle area did not show a statistically significant increase with increasing pre-slaughter weight in pigs of both weights. This also contradicts the results of publications that found a mean positive correlation ($r = 0.57$; $p<0.001$) between pre-slaughter weight and the *Longissimus* muscle area (Sládek et al., 2003) and contradicts publications where a negative weak correlation was found ($r = -0.14$; $p<0.001$) between these indicators (Barducci et al., 2020). In addition, we did not find a statistically perceptible effect of pre-slaughter weight on the *Longissimus* muscle area, contrary to the results of scientific research, which found a significant effect of pre-slaughter weight with a strength of influence 20.10% on the *Longissimus* muscle area (Andrieieva, 2020).

Our conclusions coincided with the results, which showed a low negative correlation ($r = -0.20$; $p<0.001$) between pre-slaughter weight and meat content (Sládek et al., 2003). In our experiment, the correlation between pre-slaughter weight and meat content in carcasses of 90 kg pigs was also low and negative, similar to the findings of (Barducci et al., 2020), which also indicate a negative correlation ($r = -0.23$; $p<0.001$) between these carcass slaughter indicators and a decrease in meat content with increasing pre-slaughter weight (Peinado et al. 2011). At the same time, the correlation between pre-slaughter weight and meat content was not statistically significant in carcasses of 110 kg pigs, similar to other findings (Bertol et al., 2015), which states that the meat content remains unchanged with increasing pre-slaughter weight of pigs. Thus, our results, which describe the relationship between meat content and pre-slaughter weight, agree with the data (Malgwi et al., 2022), but contradict the conclusions (Jeong et al., 2010), which show a linear increase in meat content with pre-slaughter weight increases as in carcasses 85-120 kg, and in carcasses 120–135 kg and more than 152.5 kg (Kim et al., 2005; Maiorano et al., 2007; Park, 2018). According to previous reports, the carcasses of the Irish Landrace pigs with a pre-slaughter weight 120 kg statistically perceptible outperformed the carcass meat content by 3.72% of analogues with a pre-slaughter weight 110 kg (Mykhalko et al., 2022). However, in the current experiment, on the contrary, the meat content in carcasses of light weight pigs (90 kg) was higher than in carcasses of heavy weight pigs (110 kg) by 2.7% ($p<0.001$).

Our data did not coincide with the previously published reports (Povod et al., 2020), the results of which show that in the conditions of industrial pork production it is advisable to slaughter animals with increased pre-slaughter weight more than 115 kg. It will increase carcass yield by 1.8% in gilts and by 1.6% in barrows. In our experiment, the increase in pre-slaughter weight of light pigs (90 kg) did not show a significant increase in carcass yield, and in heavy pigs (110 kg) carcass yield was significantly reduced by 0.81 kg with a decrease in pre-slaughter weight by 1 kg.

According to reports, the evaporation of moisture from the surface of pig carcasses is the cause of weight loss during the cooling period (Zhang et al., 2017). However, it was found that the pre-slaughter weight does not significantly affect the cooling losses after 24 hours in 90 kg of pigs, which coincided with widespread similar claims (Bankovskaya et al., 2016; Mykhalko et al., 2020; Povod, 2018), but contradicts the results (Patinho et al., 2013), which indicate cooling losses after 24 hours from 1.0 to 1.5% in light pigs (90 kg). However, a direct correlation was found cooling losses after 24 hours and pre-slaughter weight in 110 kg pigs, which was in direct contrast to the other findings (Daszkiewicz et al., 2011), which found that the rate of cooling losses after 24 hours was negatively correlated with pre-slaughter weight of fattening pigs.

The difference between the lengths of the bacon halves in the pig's carcasses of both groups was established. It was higher in heavy pigs (110 kg) by 3.8 cm or 4.83% ($p<0.001$). The effect of pre-slaughter weight on the length of the bacon half was confirmed by a direct and weak correlation ($r = 0.29$; $p<0.001$) in 110 kg pigs, which coincides with (Shpetny, 2018) reports, which showed a parallel increase in the length of the bacon half and pre-slaughter weight of pigs. But this contradicts the conclusions that show the absence of such effect in the carcasses of pigs 100, 110 and 120 kg (Nechmilov, 2019). However, in 90 kg pigs in our experiment, no significant effect of pre-slaughter weight on this slaughter indicator was found. This coincides with the results of experiments on the lack of correlation between the length of the bacon half and the pre-slaughter weight and coincides with the reports where is a significant impact on it pig breed (Kozir et al., 2020; Shevchuk, 2019) or diet (Fasuyi et al., 2012).

CONCLUSIONS

Increasing the pre-slaughter weight of pigs from 90 to 110 kg resulted in a decrease in meat content by no more than 5%, thus its quality class remained unchanged. An increase in the pre-slaughter weight had an effect on the increase in the carcass yield, the fat thickness and the increase in the size of the carcass and its parts, which will positively affect the volume of pork production without reducing the quality of the carcass.

We consider it necessary to continue the study of the impact on carcass quality of a further increase in the pre-slaughter weight of pigs up to 120 kg, taking into account the influence of the sex of the animals.

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RESEARCH ON THE PHYSICO-CHEMICAL ANALYSIS OF WHITE AND RED GRAPES MUST FROM A WINERY IN PRAHOVA COUNTY

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Abstract

The paper proposed an analysis of the physico-chemical composition of several types of grape varieties (white and red). Their analysis was done during the years 2019-2021 at a winery in Prahova County. The determinations were made on must, and the analyzed varieties were: Sauvignon Blanc, White Fetească and Black Fetească. The sugar content was determined, which is an important parameter in wine-making to estimate the favorable time for harvesting, but also to decide what wine is produced (dry, sweet, semi-sweet). The total acidity of the wine was also determined, acids representing important components of the wine. Low acidity will cause a "flat" and uninteresting taste, and too much acidity will cause an overly sour and astringent taste. At the same time, the pH was analyzed, which affects the taste, sugar, acidity level and stability of the wines. The observed values were in normal limits.

Key words: *acidity, must, pH, sugar, variety.*

INTRODUCTION

Viticulture represents one of the most important traditional branches of agriculture that contributed to the formation and development of civilization, as a basic occupation, bringing economic and social and cultural benefits.

The grapevine adapts very well to sloping lands, sandy or eroded soils, which are not suitable for other crops, the lands being favorable for the planting of the grapevine, having good climatic conditions and superior quality products can be obtained (Doholici, 1981). By growing vines, sloping lands are stabilized, reducing soil slips and the continuation of its solidification. In addition, it offers us many benefits (Cabernet Sauvignon, 2012).

The cultivation of vines represents an important source of profit, the production resulting from one hectare of vines is equivalent to the production obtained from ten hectares of cereal crops. Wine products are highly appreciated on the domestic and foreign market, obtaining important profits. Cultivation of vines by people living in the hill area is sometimes the only source of income. Vineyards with varieties for table grapes are exploited very well, with large productions of grapes being achieved.

Wine-viticulture products have an important contribution to the diet. Due to the high content of organic acids, mineral salts, vitamins, pectic substances, polyphenols, etc., grapes and must have a beneficial effect on the body, having an energizing, therapeutic and mineralizing effect (Doholici, 1981). Following some recent research, it was found that wine, in moderate quantities, can have an antibiotic effect on the body, especially in the cardiovascular system, preventing the causes of risk in food, caused by the excessive consumption of fats and animal proteins, which lead to favoring cardiovascular diseases (Chiva-Blanch et al., 2013, Torres et al., 2015; Snopek et al., 2018).

In some regions where wine is the traditional drink, the longevity of the population increases surprisingly (Gambini et al., 2021). The accumulation of sugars in the grains, in the form of glucose and fructose, is achieved from the reserves of the stem, the vine and from the reserves of the leaf, created daily, through photosynthesis. In the leaves, all the compounds of plant cells are born: sugars, organic acids, tannins, etc. (Boerescu, D.S., 2007). Depending on the duration of sunlight during grape ripening, a certain amount of sugar is formed through photosynthesis. Thus, the warm areas of the country will produce grapes richer in sugar, respectively, wines richer in alcohol. The migration of sugars from the woody part of the vine intensifies as the grapes enter the harvest period. The light influences the accumulation of sugars in the grapes uniformly, thus in the berries, in the peduncular area there is more sugar, and in the peripheral area of the berry it is sweeter and less acidic (Teodorescu et al., 1987).

MATERIALS AND METHODS

During the years 2019-2020, analyzes of the sugar level, acidity and pH were carried out. Determining the sugar content is an important parameter in winemaking in order to estimate the favorable time for harvesting, but also to decide what wine is produced (dry, sweet, semi-sweet). The sugar level determines an important parameter, this being the alcohol, it is estimated that at a content of 17 g/l, approximately 1 alcoholic strength is obtained, that it 10 ml of alcohol per 100 ml of wine. The sugar level of a wine is estimated to be 170-180 g/liter, and if it does not reach these limits before fermentation, sugar is added. The amount of sugar was determined with the refractometer for the must, and the unit of measure in which it was expressed is grams of sugars/1 liter of must.

The refractometer is an optic equipment which allows the measurement of refraction index of substances. It is made from an optic tube, with lentiles, which has in interior a graded scale and a glass refraction prisma, covered with a transparent plate, which moves. Thruough the glass prisma the light goes in the tube. The equipment is calibrated with distilated water and the readings are made at 20 ºC or are made the necesarry corrections. Sugar determination in must and wine are done by harvesting a sample with a pipette and putting 2-3 drops on the refractometer's prisma. The transparent lama is added and the reading are done on the graded scale at the limit point between lighted and dark field.

The acidity was determined by: the potentiometric method, mandatory in case of litigation, as a reference method; titrimetric methods in the presence of a color indicator (bromothymol blue, phenol red), as usual (indicative) methods

Excessive absorption of nitrogen and potassium is due to strong vines, which increases the pH of the grapes and the wine, which leads to spoilage of the wine quality. The pH of the must affects the taste, sugar, acidity level and stability of the wines. It is determined by the balance between the main anions (malate and tartrate) and the presence or absence of major cations (mainly potassium). The pH may increase by up to 0.2 units between harvest and delivery. High pH values can change the color of the wine. A high pH should result in wines that taste flat and red wines with a brown color. In general, a pH higher than 3.6 has a negative effect on wine quality.

RESULTS AND DISCUSSIONS

Determining the sugar content is an important parameter in winemaking in order to estimate the favorable time for harvesting, but also to decide what wine is produced (dry, sweet, semi-sweet). Depending on the amount of sugar, the following categories can exist: dry white wine - the yeasts in the dry wine consume, during fermentation, the sugar in the must and transform it into ethyl alcohol and carbon dioxide. The amount of sugar left in the composition of the wine is small, 4 g/liter; demisec white wine - sweeter than dry wine, but not sweet enough to be considered sweet, demisec wine is obtained by finishing the fermentation process longer, so that the yeasts do not consume all the sugar, and the amount of sugar remains between 4 and 12 g/liter; sweet wine - the amount of sugar is between 12 and 50 g/liter, the wine is semi-sweet, being obtained by stopping the fermentation of the must before it is complete; sweet white wine - the sugar concentration in sweet wine is 50 g/liter.

The total acidity of the must is determined by the sum of the functions of free and semi-bound acids, which can be titrated when the pH is brought to the value of 7 by adding a titrated alkaline solution. Acids are important components of wine. Low acidity will cause a "flat" and uninteresting taste, and too high acidity will cause a too sour and astringent taste.

The excessive absorption of nitrogen and potassium is due to strong vines, which increases the pH of the grapes, leading to the deterioration of the quality of the wine. The pH of the must affects the taste, sugar, acidity level and stability of the wines. It is determined by the balance between the main anions (malate and tartrate) and the presence or absence of major cations (mainly potassium) influences the pH of the wine, the time and management of the harvest. The pH may increase by up to 0.2 units between harvest and delivery. High pH values can change the color of the wine. A high pH should result in wines that taste flat and red wines with a brown color. In general, a pH higher than 3.6 has a negative effect on wine quality.

The White Feteasca variety is one of the most valuable Romanian grape varieties. It produces dry or semi-dry wines, with balanced alcohol content (11.5-12% strength) and acidity, characterized by great finesse, but also semi-sweet, sweet and sparkling wines. White Feteasca produces a fine, elegant, round, velvety, ample, full-bodied wine.

It has a very high sugar accumulation power, from this point of view being among the first varieties in the world. The amount of sugar accumulated in grapes is normally 180-220 g/l of must, but it can reach up to 240-250 g/l and even 270 g/l, in overripe conditions.

For white Feteasca, for the years studied (2019-2021), the sugar, acidity and pH values can be found in the Tables 1, 2 and 3.

From the analyzed of these data, it can be seen that the largest amount of sugar was accumulated towards the end of the harvest period, respectively on September 2 and 5. Also, the highest acidity reached 12.5 g/l in the first days of harvesting, decreasing during the harvest (Table 1).

Table 1. Sugar content, acidity and pH of the White Fetească variety in 2019

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
12.08	122	12.2	2.86
19.08	170	7.6	3.06
22.08	172	5.7	3.12
26.08	183	5.2	3.11
29.08	188	4.3	3.18
02.09	210	3.7	3.44
05.09	210	3.8	3.55
Average	**179.29**	**6.07**	**3.19**

Also, in 2020, the amount of sugar accumulated was towards the end of the harvest period, the highest acidity 6 g/l was recorded at the beginning of the harvest period, respectively on 6.08, the pH maintaining between values between 3.08-3.46 (Table 2).

Table 2. Sugar content, acidity and pH of the White Fetească variety in 2020

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
06.08	135	6	3.08
13.08	151	4.7	3.12
17.08	162	4.1	3.2
20.08	167	4.2	3.25
24.08	175	3.7	3.33
27.08	175	3.5	3.46
Average	160.83	4.36	3.19

In the last year of the study, regarding the White Fetească variety, the recorded sugar value was 143 g/l on August 23, reaching a maximum of 204 g/l towards the end of the harvest. Regarding the pH, the values were between 2.8-3.47 (Table 3).

Table 3. Sugar content, acidity and pH of the White Fetească variety in 2021

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
23.08	143	4.8	3.06
26.08	156	3.9	2.8
30.08	164	3.3	2.89
02.09	162	3.6	3
06.09	183	3.4	3.29
09.09	183	3.7	3.3
13.09	191	3.2	3.47
14.09	204	3.3	3.32
Average	173.25	3.62	3.14

The Sauvignon variety ranks second after Chardonnay in international trade. This is originally from Bordeaux, which is located in France and was introduced to Romania in the last part of the last century. To avoid confusion with Cabernet Sauvignon, producers write Sauvignon Blanc on the label.

The wines obtained from Sauvigon Blanc are white wines of superior quality. This variety can be vinified to obtain dry, semi-dry or sweet wines. Sauvignon Blanc is often overlooked by experts because the wines produced from this variety are relatively simple, have strong vegetal notes and do not have great aging potential. However, Sauvignon Blanc has a much more complex and attractive character than it suggests at first sight or tasting. The variety can be subjected to various styles of winemaking, the result being wines that differ in taste, appearance and personality.

In the Sauvignon Blanc variety, the analyzed values regarding the sugar content ranged between 111 g/l in the first harvest period, reaching 217 g/l in the last period. Regarding pH, the values ranged between 2.8 and 3.25 (Table 4).

Table 4. Sugar content, acidity and pH of the Sovingnon Blanc variety in 2019

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
12.08	111	18.6	2.75
18.08	156	13	2.8
22.08	162	9.6	2.91
26.08	212	8.3	3
29.08	212	7.3	3.01
02.09	215	6	3.25
05.09	217	5.3	3.3
Average	**153.28**	**9.72**	**3.00**

In 2020, the Sauvignon Blanc variety had sugar values between 167 g/l, increasing recently to 230 g/l. Throughout the period, the acidity had an average of 5.75, and the pH 3.28.

Table 5. Sugar content, acidity and pH of the Sovingnon Blanc variety in 2020

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
20.08	167	7.8	3.06
24.08	175	6.3	3.2
27.08	215	5.9	3.29
03.09	204	6	3.3
07.09	194	4.5	3.38
10.09	230	4	3.48
Average	**197.5**	**5.75**	**3.28**

From the analysis of the data below we can see that the highest sugar content was 228 g/l recorded in September, the highest acidity was in the first period of the harvest 6.6, it will decrease until the end of the period to 4.6 g/l. The pH recorded values between 3 and a maximum of 3.27 (Table 6).

Table 6. Sugar content, acidity and pH of the Sovingnon Blanc variety in 2021

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
06.09	172 g/l	6.6 g/l	3.16
09.09	178 g/l	6.4 g/l	3
13.09	196 g/l	6.2 g/l	3.2
14.09	209 g/l	6.2 g/l	3.29
23.09	228 g/l	4.6 g/l	3.27
Average	**196.6**	**4.76**	**3.18**

The Black Fetească variety is an autochthonous variety, cultivated for a long time on large areas in the southern part of Moldova and in the eastern area of Muntenia. From this variety, you can get both special rosés and red wines that can be aged in quality wood and later in glass, resulting in high-brand wines with a pronounced typicality. Depending on the winemaking techniques, wines with varied aromas and fine tannin structure, good acidity, medium to full body and a medium to high alcohol level can be obtained. Black Fetesca offers a dry or demi-dry top quality red wine, ruby-red in color, with an alcoholic strength of 12-13% with a higher acidity, the young wine is very harsh, but once it ages, it becomes balanced. In 2019, the picking of the Back Feteasca variety took place between August and September over the course of three days. The Black Fetească variety had an average value of 242.66, acidity of 6.13 and a pH of 3.56 (Table 7).

Table 7. Sugar content, acidity and pH of the Black Fetească variety in 2019

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
26.08	236	7.2	3.24
02.09	242	6.7	3.31
05.09	250	4.5	3.52
Average	**242.66**	**6.13**	**3.56**

Table 8. Sugar content, acidity and pH of the Black Fetească variety in 2020

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
02.09	234	5.4	3.37
05.09	238	5.2	3.44
21.09	240	4	3.32
Average	**237.33**	**4.86**	**3.37**

In the last year of the study, the recorded averages were 186.6 for sugar, acidity recorded an average of 5.82, and pH 3.15 (Table 9).

Table 9. Sugar content, acidity and pH of the Black Fetească variety in 2021

Analysis data	Type of analysis		
	Sugar (g/l)	Acidity (g/l)	pH
02.09	160	6.1	2.8
06.09	170	6.3	3.3
09.09	194	5.8	3.2
13.09	199	5.9	3.2
16.09	210	5	3.26
Average	**186.6**	**5.82**	**3.15**

CONCLUSIONS

Full maturity represents the moment when carbohydrates, total acidity and 1000 berry weight achieve the characteristic levels of the varieties. The evolution of the weight of the berries gives indications on the qualitative side, the variation of the content in sugars and acidity, as well as the establishment of the ratio in which they are found give indications especially on the qualitative side. So, by full maturity is meant that moment when the grape has accumulated a maximum amount of carbohydrates without its weight having decreased, and the acidity is moderate. White grapes turn yellowish and black ones turn light black. The period can be limited to two weeks, observing a sudden increase in the sugar content.

In this study, the evolution of the physico-chemical analyzes of white and red grape varieties was followed during the years 2019-2021 in a winery in Prahova county.

From the statistics, it is found that in 2019, a higher level of sugar in the red varieties, compared to the white one, and the acidity level of the pH is higher in the white grapes compared to the black ones. It was found that in 2020 the red variety was much richer in terms of the level of sucrose in the grapes, and the white variety in acidity and pH. The year 2021 also saw a very good harvest in terms of the red variety, both in terms of sugar level and acidity and pH. The white variety had normal values throughout the years of study. The accumulation of sugars in the grains, in the form of glucose and fructose, is made from the reserves of the stem, the vine and from the reserves of the leaf, created daily, through photosynthesis. All the compounds of plant cells are born in the leaves: sugars, organic acids, tannins, etc. Depending on the duration of sunlight during grape ripening, a certain amount of sugar is formed through photosynthesis. Thus, the warm areas of the country will produce grapes richer in sugar, respectively, wines richer in alcohol. The migration of sugars from the woody part of the vine intensifies as the grapes enter the fallow period. The light influences the accumulation of sugars in the grapes uniformly, thus in the berries, in the peduncular area there is more sugar, and in the peripheral area of the berry it is sweeter and less acidic. The decrease in acidity is the reverse of the process of accumulation of sugars. In green grapes, the acidity is 20 g sulfuric acid/l, so that in a few weeks it drops to 4-6 g sulfuric acid/l.

The dynamics of titratable acidity is due to the evolution of tartaric acid and malic acid. Tartaric acid undergoes minimal changes, and malic acid, because it is more labile, will quantitatively decrease faster in the first stage and then more slowly, depending on the ripening of the grapes. Varieties are differentiated by their content in malic acid, there are varieties rich in malic acid and varieties poor in malic acid. During the ripening phase of the grape, the maturity index is monitored, which represents a sugar/acidity ratio, which, depending on the pedoclimatic conditions, varies between 30 and 70.

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ELECTROACTIVATION EMERGING METHOD OF PROCESSING OF WHEY WITH HIGH PROTEIN CONTENT

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Abstract

Electroactivation is an emerging method of non-residual processing of secondary dairy products, namely, whey with high protein content, which presents one of the directions that describe the treatment of whey with different initial protein content and the extraction of serum proteins into protein mineral concentrates. The treatment of whey with high protein content was carried out in different electrolyzers with different ratios of the processed whey volume to the electrode/cathode surface and different geometric shapes, different distances between the electrodes and the membrane, which influences the specific energy consumption per unit volume. The main objective of the work was the maximum extraction of whey proteins in protein mineral concentrates and the simultaneous isomerization of lactose into lactulose at low energy consumption and the exclusion of "dead"/inefficient areas for the electroactivation of whey in different diaphragm electrolyzers. The mechanisms of whey protein extraction depending on pH and redox potential values upon electrochemical activation of whey with high protein content are presented.

***Key words**: electroactivation, pH, protein mineral concentrates, redox potential, whey.*

INTRODUCTION

The factors that influence industrial development are technological innovations, economic comfort, and environmental safety. In the context of the circular economy, regarding the processing of waste/secondary products, the complex, non-residual treatment is the main factor that satisfies the consumer and governmental requirements, which involves the initiation of extensive research, for the correction of certain actions applied in the technology (Ebrahim, 2020; Wang et al., 2021). Our society is currently facing the negative impact affecting the environment. Sustainable development implies the solution of ecological and social problems that require the implementation of emerging and innovative technologies (Bordean, 2009).
The use of electrochemical methods, in particular, electroactivation, allows solving a number of ecological problems, beneficial in alternative industrial processes, environmental protection, and pollution monitoring. Electrotechnologies are supposed to be among the most harmless ones in the processing of secondary products (Bersier et al., 2011; Ghazouani et al., 2019, Marin et al., 2017).
Electroactivation as a sustainable method for processing dispersed media, in particular, secondary dairy products (whey, buttermilk, etc.) is an alternative to conventional methods and is of growing interest due to its ability to transform electrical energy into chemical energy.
The principle of electroactivation can be defined as the intensification of the electron donor-acceptor properties through the energy exchange of the solution and the substances produced at the electrodes. The electrochemical activation method is a process of activation of water molecules caused by an electric field, which induces their metastable state, causing changes in pH, redox potential (E, mV), and electroconductivity (Karim & Aider, 2020; Kareb et al., 2017; Liato et al., 2015).
The global amount of obtained whey is estimated at approximately 180-190·10^6 tons/year, of which only 50% is processed (El-Tanboly et al., 2017; Dimou et al., 2019).

About 50% of the world production of whey obtained after cheese making is treated and transformed into various food and fodder products. Half of that amount is used directly in a liquid form, 30% as whey powder (obtained by spraying), 15% as lactose and its derivatives, and the rest - as protein concentrates obtained by membrane processes (Buchanan, 2023).

Membranes in whey processing play an important role in the separation of proteins and other valuable substances from whey, which have two aspects of use: filtration by the pore size (microfiltration, ultrafiltration, nanofiltration, etc.) and the use of ion-selective membranes at the electrodialysis and electroactivation of whey (Paladii et al., 2021a).

A total of $40 \cdot 10^6$ tons/year is produced in the European Union, the annual surplus of whey is $13 \cdot 10^6$ tons, containing approximately 619,250 tons of lactose (Zotta et al., 2020; Buchanan, 2023).

The solid content of whey (7-8%) makes up 50-70% of that of the initial milk. Almost all lactose and the most precious protein fractions (β-lactoglobulins, α-lactalbumins, bovine serum albumin, immunoglobulins, etc.) pass into the whey; they are not retained in primary dairy products, nor are a number of macro- and microelements, and vitamins (Paladii et al., 2021b; Soltani et al., 2017).

Proteins, due to their biological and nutritional properties, show increased interest in their usage in both food and pharmaceutical industries. A high potential of whey as a valuable raw material for obtaining food and bioactive substances with added value generates new directions in the development of technologies for the reuse of by-products of the dairy industry (Tsutsumi, 2014; Patricia et al., 2019).

The non-residual processing of whey and the valorization of its protein content is a complex problem from a technical and technological point of view, however, being of major importance.

The extraction of whey proteins from different types of whey ensures the reduction of the harmful impact on the environment, using the internal forces of the processed product, based on multiple inter- and intramolecular reactions.

Electroactivation is carried out via the electrical action on a technological liquid in the region of the polarized electrode, for example, in a diaphragm electrolyzer. However, unlike electrolysis and electrodialysis, electroactivation does not present a finite chemical process and is used to regulate the reactive capacity of the physico-chemical properties of media in various technological processes, with the aim of optimizing and increasing the effectiveness of the proposed process (Vrabie et al., 2019).

The electroactivation of whey is an amazing treatment process, that allows simultaneously the isomerization of lactose into lactulose and the recovery of protein mineral concentrates (PMC) (Bologa et al., 2009).

The purpose of the present work was to discuss the non-residual processing of whey with a high protein content, which allows the recovery of protein mineral concentrates at different treatment conditions.

MATERIALS AND METHODS

The experimental model of an electrolyzer

The electroactivation of whey with a high protein content imposes certain technical requirements to ensure the command and control of the technological process (see used electrolyzers in Figures 1-2) (Vrabie et al., 2019).

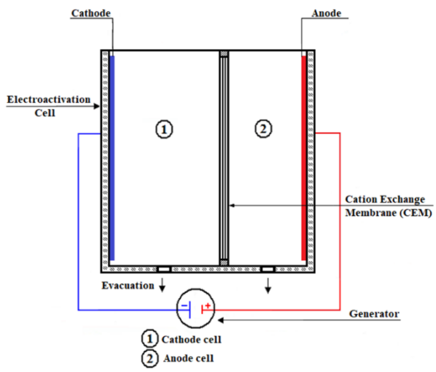

Figure 1. The layout of experimental membrane electrolyzers EDP-2 and EDP-4

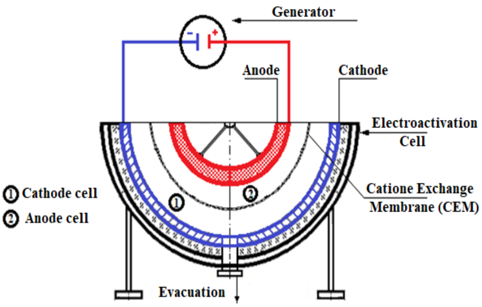

Figure 2. The layout of experimental membrane electrolyzers EDC-3 and EDC-pilot

The results of the research regarding the establishment of the factors that influence the electroactivation of whey, the understanding of the physico-chemical and biochemical processes that take place when the electric current passes through a dispersed medium with a complex biological structure such as whey, allowed the development of the principles and the constructive layouts of certain electrolyzers with different geometrical configurations, adapted to the peculiarities and technological requirements of the processing of secondary dairy products with the extraction of PMC and the simultaneous isomerization of lactose into lactulose.

Different types of electrolyzers (conventionally called EDP-2, EDP-4, EDC-3, and EDC-pilot) with certain geometric parameters, allowing the non-residual processing of whey, were fabricated and used in the research (Figure 3).

The EDP-2 and EDP-4 electrolyzers developed in the form of a parallelepiped (Figure 1) have the same distance between the electrodes and between the electrodes and the membrane, but a different ratio of the processed whey volume (V) to the electrode surface (S) V/S; for EDP-2 this ratio is 1.4 and for EDP-4 – 1.0 (Figures 3, 4).

The EDC-3 and EDC-pilot electrolyzers were intended for the processing of whey in a periodic and continuous regime of the flow rate of whey and the secondary liquid (anodic liquid).

They have the semi-cylindrical dielectric housing with the anode and cathode cells, the membrane, located on the semi-cylindrical housing, the electrodes: the cathode, placed on the semi-cylindrical casing and the anode, the inlet and outlet valves of the anodic liquid, the inlet and outlet valves of the whey.

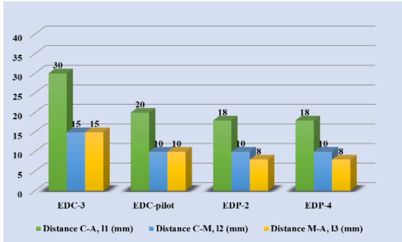

Figure 3. Constructive parameters of electrolyzers EDC-3, EDC-pilot, EDP-2, and EDP-4): l_1 - cathode (C) and anode (A) distance; l_2 - cathode and membrane distance (M); l_3 - membrane and anode distance

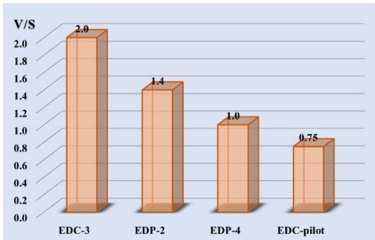

Figure 4. Variations of the ratio of the processed whey volume (V) to the surface of the electrode (cathode) (S) V/S of the electrolyzers EDC-3, EDC-pilot, EDP-2, and EDP-4

They have different distances between the electrodes and between the electrodes and the membrane, and the ratio of the processed whey volume (V) to the electrode surface (S) V/S, so that for EDC-3 this ratio is 2.0 and for EDC-pilot - 0.75 (Figures 3, 4).

The electroactivation of whey was carried out in different electrolyzers at different densities of the electric current: 10 and 20 mA/cm^2, keeping them constant during the processing period. The pumping regime of the working liquid (different types of whey in the cathode cell - CC) and the secondary liquid (2% $CaCl_2$ solution in the anode cell - AC) is periodic in nature. PMC were collected at certain treatment periods. The processed whey was discharged as foam, mixed to destroy the foam formed during processing and separated in the form of PMC as sediment and deproteinized whey (DW) as supernatant, in the field of mass forces. The secondary liquid from the anode cell underwent recycling.

The research was carried out using whey with a high protein content (supplied by the "JLC" Joint Stock Company, Chisinau, RM) obtained after the production of the granulated cheese "Grăuncior". The protein content was

determined by the Warburg method with the СФ-56 spectrophotometer (standard solution - bovine serum albumin, calibration coefficient k=1.72) (Ressler, 1976).
The amount of protein extracted in PMC (or the degree of protein extraction in PMC) (Q, %), was calculated by the difference between the protein content in the initial whey (IW) and that remaining in DW:

$$Q = Q_{IW} - Q_{DW}, \%$$

Where: Q is the protein content in PMC, Q_{IW} is the protein content in the IW, and Q_{DW} is the protein content in the DW.

RESULTS AND DISCUSSIONS

The electroactivation of whey with a high protein content obtained after the production of the granulated cheese "Grăuncior" processed in different electrolyzers allows the extraction of whey proteins into PMC and the simultaneous isomerization of lactose into lactulose.
The processing was carried out at different densities of electric current: 10 and 20 mA/cm^2, kept constant during the treatment; the variations of voltage, temperature, and physico-chemical parameters (pH values and redox-potential) were recorded, then the degree of the whey proteins extraction in PMC was specified.
The determination of the specific energy consumption per unit volume is a decisive factor in the study of the electroactivation of high-protein whey processed in different electrolyzers, namely: the extraction of whey proteins in PMC and the isomerization of lactose into lactulose occur simultaneously, so, at the specific consumption of energy, a possibility of obtaining two products at the same time - protein concentrates and the DW, which contains isomerized lactulose, appears.
The variations of the specific energy consumption per volume unit, A/V (Wh/ml), when electroactivating whey with a high protein content, in the investigated electrolyzers, indicate the lowest values when treated in the EDC-3 electrolyzer, which allows the processing of whey with the lowest specific energy consumption per unit volume at both 10 and 20 mA/cm^2 current densities in the processing regimes (Figures 5, 6).

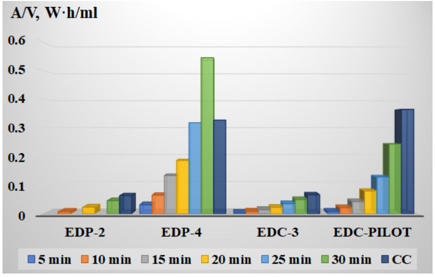

Figure 5. Variations of the specific energy consumption per unit of volume, A/V (Wh/ml), in EDP-2, EDP-4, EDC-3, and EDC-pilot electrolyzers, during the electroactivation of the whey obtained after the production of "Grăuncior", at j = 10 mA/cm^2

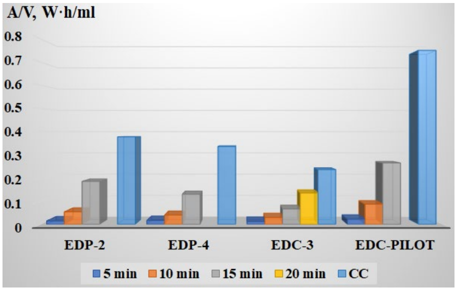

Figure 6. Variations of the specific energy consumption per unit of volume, A/V (Wh/ml), in EDP-2, EDP-4, EDC-3, and EDC-pilot electrolyzers, during the electroactivation of the whey obtained after the production of "Grăuncior", at j = 20 mA/cm^2

The variations of the consolidated specific energy consumption per unit of volume during the electroactivation of whey with a high protein content, in the periodic treatment regime at the electric current densities of 10 and 20 mA/cm^2 in different electrolyzers indicate the rationality of the use of EDC-3 (Figure 7).
The electroactivation of the whey investigated in this article in different electrolyzers with different geometrical parameters influences the specific energy consumption per unit of volume, and, namely, it depends on the processed volume, the initial solid and protein content of this type of whey and is attributed to obtaining two products simultaneously: PMC with different content of whey proteins and the DW containing isomerized lactulose.

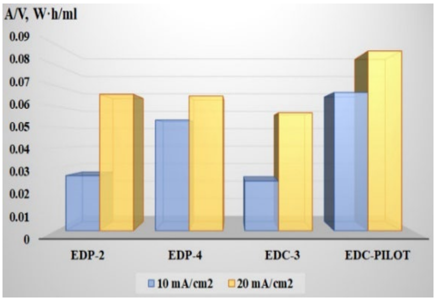

Figure 7. Variations of A/V (Wh/ml) during electroactivation of whey obtained after the production of "Grăuncior" in electrolyzers EDP-2, EDP-4, EDC-3, and EDC -pilot at the electric current densities 10 and 20 mA/cm^2

The variations of thermal parameters during the electroactivation of whey in different electrolyzers requires the correct control of processing regimes, which is caused by Joule heating, dependent on the conductivity of the treated medium.

The necessary conditions for reducing the resistance of the system require: ensuring the presence of charge carriers depending on the solid content of whey, reducing the membrane resistance, maintaining the optimal distance between the electrodes and between the electrodes and the membrane.

The whey proteins have a thermal denaturation point (55-60°C), and their extraction into protein concentrates requires compliance with certain processing regimes.

The main physico-chemical parameters - pH values and redox potential E, (mV) - during the electroactivation of whey with a high protein content in different electrolyzers vary due to the characteristic reactions of the water dissociation process in the cathode and anode cells at the surface of the electrodes, and they characterize the physical, chemical, and biochemical changes of whey.

Depending on the reaction of the medium and the ratio of the acidic or alkaline radicals of amino acids, proteins have a positive or negative charge.

Simultaneously, upon electroactivation, the dissociation of water at the cathode forms hydroxide ions, which characterizes the alkaline medium and releases hydrogen gas, which imposes ionic flotation through foaming, accompanied by protein salinization due to the migration of bivalent ions from the anode cell, which intensifies the foaming process and the "unfolding" of protein molecules.

The amine groups, in turn, intensify the alkaline medium, imposing a rapid increase of pH values in the cathode cell, which favors the passage of protein fractions through their isoelectric point and a considerable decrease in the redox potential, characterized by intense reduction reactions.

An increase of the pH values upon electroactivation of dispersed media is also accompanied by the passage of aquacomplexes, where polarized water molecules that maintain the colloidal structure serve as ligands together with proteins, in hydrocomplexes, that have hydroxyl ions as ligands (Vrabie et al., 2011).

Depending on both the protein content of the whey and the geometric shape of the electrolyzer and the volume processed, the transition of aquacomplexes into hydrocomplexes is different.

In the same way, the redox potential decrease characterizes the redox reactions which have intensely negative values in the cathode cell, where, respectively, reductive reactions characteristic of the processed medium take place.

A slower increase of the pH values in electrolyzers EDP-4, EDC-3, and EDC-pilot upon electroactivation of whey with a high protein content, at electric current density j = 10 mA/cm^2, denotes a slower transition of aquacomplexes into hydrocomplexes compared to those in EDP-2 in which this passage is practically absent, and the formation of hydrocomplexes occurs very quickly (Figure 8).

The redox potential decrease has the same character in the four electrolyzers, indicating the rate of formation of active reductants (Figure 9).

The analysis of the physico-chemical parameters (pH and E, mV) allows the investigation of the multiple transformations of the main components of whey, first of all, of the protein fractions, as well as the activation state of the amino acids, which depends on the energy delivered to the system and their state activation.

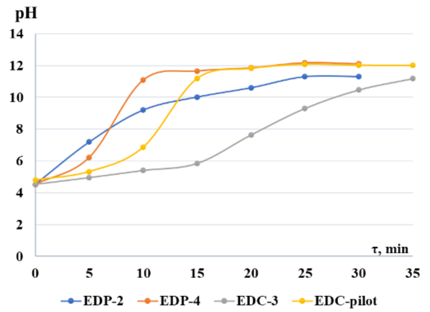

Figure 8. Variations of pH in electrolyzers EDP-2, EDP-4, EDC-3, and EDC-pilot during electroactivation of whey obtained after the production of "Grăuncior", at j = 10 mA/cm^2

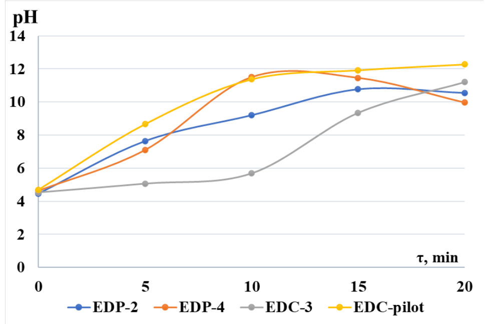

Figure 10. Variations of pH in electrolyzers EDP-2, EDP-4, EDC-3, and EDC-pilot during electroactivation of whey obtained after the production of "Grăuncior", at j = 20 mA/cm^2

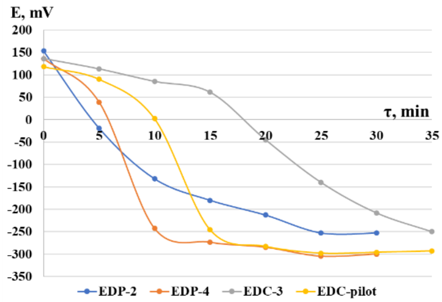

Figure 9. Variations of redox potential (E, mV) in electrolyzers EDP-2, EDP-4, EDC-3, and EDC-pilot during electroactivation of whey obtained after the production of "Grăuncior", at j = 10 mA/cm^2

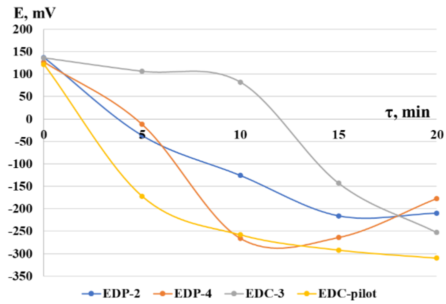

Figure 11. Variations of redox potential (E, mV) in electrolyzers EDP-2, EDP-4, EDC-3, and EDC-pilot during electroactivation of whey obtained after the production of "Grăuncior", at j = 20 mA/cm^2

The variations of the pH values in electrolyzers EDP-2, EDP-4, EDC-3, and EDC-pilot during the electroactivation of whey with a high protein content, at the electric current density j = 20 mA/cm^2, indicate to a faster transition of both aquacomplexes into hydrocomplexes (Figure 10), as well as the rate of formation of active reductants, characterized by redox potential variation (Figure 11).

The exception is EDC-3 which has a larger processing volume and a longer distance between the electrodes and between the electrodes and the membrane.

The electroactivation of this type of whey with a high protein content requires the specific energy consumption to be lower, thus ensuring the achievement of the maximum necessary for the extraction of whey proteins under optimal processing conditions.

The extraction of whey proteins in PMC (Q, %) from the IW varies depending on the initial solid content, the processing regime (different current densities, the amount of IW processed), the variations of electrical, thermal, and physico-chemical parameters, for the duration of the treatment. The intense foaming in the first minutes of processing is an indication of an intense salinization of the protein fractions, which leads to the formation of PMC.

The salinization of whey proteins upon electroactivation is one of the multiple mechanisms that contribute to the extraction of protein fractions in PMC.

The variations of the degree of extraction (Q, %) of whey proteins in PMC upon electroacti-vation of whey with a high protein content, at the electric current density j = 10 mA/cm^2, in electrolyzers EDP-2, EDP-4, EDC-3, and EDC-pilot, indicates the highest values for electroactivation in EDC-3, making up about

80% both in the foam phase (30 min of treatment) and in the liquid phase - the content of the cathode cell, and is considerably higher compared to that in three other electrolyzers (Figure 12).
The variations of the degree of extraction (Q, %) of whey proteins in PMC upon the electroactivation of whey with a high protein content, at the electric current density j=20 mA/cm^2, shows that in the EDP-2, EDP-4, and EDC-pilot electrolyzers many whey proteins in PMC are extracted, but in EDC-3, during the entire treatment period, a lower percentage of extraction occurs compared to electroactivation at 10 mA/cm^2, which is possibly influenced by the hydrolysis of serum proteins, which are not extracted in PMC, but remain in the DW and allows us to conclude that at a higher electric current density under these treatment conditions protein mineral hydrolysates (PMH) in EDC-pilot could be obtained (Figure 13).

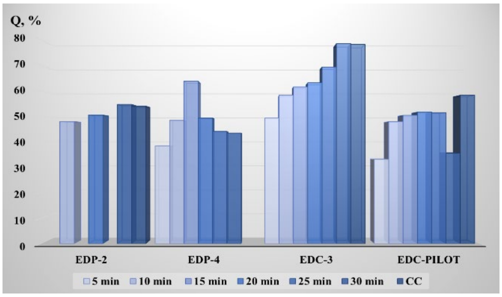

Figure 12. Variations of the degree of extraction (Q, %) of whey proteins in PMC upon electroactivation of whey obtained after the production of "Grăuncior", at j=10 mA/cm^2, in electrolyzers EDP-2, EDP-4, EDC-3, and EDC pilot

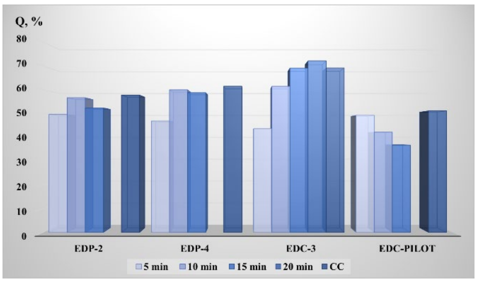

Figure 13. Variations of the degree of extraction (Q, %) of whey proteins in PMC upon electroactivation of whey obtained after the production of "Grăuncior", at j = 20 mA/cm^2, in electrolyzers EDP-2, EDP-4, EDC-3, and EDC pilot

The different and inhomogeneous extraction of serum proteins in PMC during the electroactivation of whey with a high protein content in different electrolyzers is primarily conditioned by the properties of each individual fraction and their behavior during electrochemical activation, the initial solid content, especially the protein one, of the constructive/geometrical parameters, of the processing regimes, of the electrical, thermal, and physico-chemical parameters that denote the individual approach of the investigated whey depending on the initial solid content, which requires the stipulation of the technical and technological treatment conditions.
It is important to adjust the technical and technological parameters in accordance with the characteristics of the specific energy consumption per unit of volume, which denotes the efficiency and profitability of the electroactivation of different types of secondary dairy products.

CONCLUSIONS

The need for non-residual processing of whey and the valorization of its protein content presents a complex technical and technological problem of major importance.
The electroactivation of whey with a high protein content, at electric current densities j = 10 and 20 mA/cm^2, in electrolyzers with different constructive/geometric configurations (named EDP-2, EDP-4, EDC-3, and EDC-pilot) allowed the formulation of the following conclusions:
1. The main factors that influence the electroactivation of whey are: constructive/geometrical parameters (with parallelepiped or semi-cylindrical casing); the volume of the processed whey and the V/S ratio; the distance between the electrodes and between the electrodes and the membrane; the electric current density (j = 10 and 20 mA/cm^2); the solid content of the IW, which characterizes the specific energy consumption per unit of volume A/V (Wh/ml).
2. The different and inhomogeneous extraction of whey proteins in PMC at the electroactivation of high-protein whey in different electrolyzers and different processing regimes is conditioned by the properties of each

individual fraction and their behavior at the electrochemical activation, the initial solid content, in particular, of the protein one.

3. The maximum extraction of whey proteins in PMC takes place at the electric current density j = 10 mA/cm^2 in electrolyzers with a semi-cylindrical casing, which allows the exclusion of "dead"/inefficient areas (EDC-3 and EDC-pilot) - about 80% and 60%; at the electric current density j = 20 mA/cm^2, the extraction is less and, possibly, influenced by protein hydrolysis and allows us to conclude that at a higher electric current density under these treatment conditions PMH could be obtained in the EDC-pilot electrolyzer.

4. The exclusion of "dead"/inefficient areas when using the EDC-3 electrolyzer allows the maximum extraction of whey proteins in PMC at a specific energy consumption twice lower.

5. The recovery of whey proteins at different pH and redox potential values at the electrochemical activation of high protein whey is due to the sedimentation of different protein fractions at their isoelectric point and salinization of whey proteins.

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CURRENT TRENDS IN THE DEVELOPMENT OF VALUE-ADDED FOOD PRODUCTS BY EXPLOITING THE FUNCTIONAL POTENTIAL OF CHESTNUT FLOUR AND ROSEHIP POWDER

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Abstract
The scientific research done in the past few years has been conducted with the aim of discovering and developing new materials and technologies to develop foods that are healthier and more valuable nutrition-wise. The new generation of products should be in accordance with the environmental directives, sustainable, should do no harm, and should also ensure equitability of the market. Among the unconventional materials that caught our attention, chestnuts and rosehips are sustainable, underexploited resources that meet dietary and nutritional requirements. The aim of this paper is to gather comprehensive knowledge and get an overview of the use of chestnut flour and rosehip powder in different value-added food products, and to identify the possible gaps in the current knowledge. This work will help the further research in the field of valorization of chestnut and rosehip, and therefore, contribute to the development of innovative functional food formulations. Moreover, this matter is of importance because the generation of novel products would meet consumer expectations and will both help generate income to the food industry and provide a more sustainable use of resources.

Key words: *chestnut flour, functional foods, nutritional properties, rosehip powder, value-added food product.*

INTRODUCTION

Numerous uncertainties regarding the future of food and agriculture in all of its sectors (crops, animals, fisheries, and forests) have led to major issues and worries about sustainability and performance (Gonciarov et al., 2015). A lot of factors influence uncertainty, including population increase, dietary preferences, technological advancements, income distribution, the condition of natural resources, climate change, etc. (FAO, 2018). The world's population is constantly growing, while the Earth's ability to renew its resources is declining constantly. As a result, the bioresources needed for food production are also diminishing, and for this reason, new approaches are needed in order to feed the current and future global population.

At the same time, in the past years, the population has been showing interest in food and nutrition, not only as a way of eating a certain amount of food to survive, but rather as an instrument in disease prevention, bettering physical and mental well-being, and even to slow down the aging process.

This topic is important firstly because consumers need and expectations must be met, this resulting in a growth in capital for the food industry, and secondly, because new approaches are needed in the field in order to develop more sustainable food developing processes, to stop waste and to discover new materials that can enhance human food products, while keeping in mint the health and environmental aspects.

Foods with additional health benefits, beyond the regular nutritional qualities, are more appealing to consumers. As a result, functional foods are more in demand right now (Petcu et al., 2023). People's hectic lives today prevent them from adopting healthier eating habits, making them more vulnerable to conditions like diabetes, obesity, cardiovascular disease, and high blood pressure. As a result, consuming foods high in dietary fiber and antioxidants will help reduce these health problems (Predescu et al., 2018). In recent years, demand in food products with added value has increased. Numerous studies, including those cited in this review, have demonstrated how scientists and researchers in the field of nutrition are looking for ways to enhance food products by fortifying and enriching them with various components and additives that are advantageous to human health (Goncearov et al., 2004).

As it has been proven through numerous investigations, chestnuts and chestnut by-products have been proven to have a great impact on health, sustainability, and waste management. Food products containing chestnuts and chestnut flour are valuable when it comes to nutritional and functional properties, as we can see from the results shown by different studies that were conducted by researchers around the world.

Innovative and fortified food products are absolutely necessary. Consumers have demonstrated that they are becoming increasingly interested in nutrition as well as food as a topic that goes beyond simply eating for survival. Therefore, it is obvious that the market requires an entirely novel category of edible goods.

Products that utilize unconventional materials are becoming a priority in research. The use of these materials for the development of food products has multiple benefits. In addition to the fact that the products created have an enriched nutritional value, certain population groups have opened a door to products that they could not have consumed before. People who have certain purposes regarding physical form, and who, for example, avoid consuming products that have a high sugar content, can find alternatives for sweet products that do not contain sugar. People with celiac diseases, who cannot enjoy traditional pastry and confectionery, can choose products based on flour that is not wheat, but coconut, or chestnut.

A healthy diet helps to avoid diseases, extend life, boost productivity, and foster circumstances for the body to properly adapt to its surroundings. Additionally, one of the best ways to boost the competitiveness of food industry manufacturers is to create a variety of specialized goods that are filled with food ingredients at a level that satisfies the physiological requirements of the body. Although the chestnut has recently come to light as a raw resource, its advantages are still not fully discovered. There is undoubtedly more to learn about this nutrient-dense fruit, and additional research on chestnuts and their qualities is required.

In line with the above considerations, our work will address several key points regarding the sustainable valorisation of chestnuts and rosehips, the nutritional aspects of chestnuts flour and rosehip powder, the impact of exploiting the nutritive and functional properties of chestnuts and rosehips on human health, as well as the currents trends registered in the use of chestnuts flour and rosehip powder to develop value-added food formulas.

CHESTNUTS AND CHESTNUT FLOUR

Southern Europe, Asia (China), and the Americas are home to the Fagaceae species *Castanea sativa* Mill. Chestnuts are a seasonal nut (fall) in Mediterranean nations, yet they are highly prized globally (Cruz et al., 2013). A significant species of trees with a priceless historical and cultural legacy, the European or sweet chestnut, is crucial to the financial and environmental context of mountainous regions. *Castanea crenata* Sieb. and Zucc. is common in Japan, *Castanea mollissima* Bl. is found in Korea and China, and *Castanea dentata* Borkh is present in North America (Cruz et al., 2013). Other species are also prevalent in these regions of the world.

Due to their starch, carbs, and low fat content, chestnuts can be employed as a significant source of nutritive energy (Vasconcelos et al., 2010). The majority compound is water, with humidity ranging from 40 to 64 g/100 g of fresh chestnut weight. Due to the high likelihood of mold growth and the large weight loss during storage, this high moisture content is a significant drawback for long-term preservation (Savu & Petcu, 2002). Chestnuts are primarily made up of carbohydrates (75-91%) on a dry basis, particularly starch (39-82%). The exact contents of amylose and amylopectin, which make up roughly 33% and 67% of the starch content, respectively, have been the subject of numerous investigations

(Pizzoferrato et al., 1999; Attanasio et al., 2004; Pereira-Lorenzo et al., 2006).
According to Pizzoferrato et al. (1999), starch can have a positive impact on intestinal functions due to the existence of short-chain fatty acids produced by the bacterial catabolism of dextrins obtained from amylopectin as well as by administering energy generated by the catabolism process of amylosis and amylopectin in glucose.
Chestnuts also contain fiber, which is a crucial ingredient. Dietary fiber has a good impact on health. Proteins vary between 3.9 to 10.9 g/100 g of dry mass, with a number of essential amino acids being recognized, such as arginine (Arg), phenylalanine (Phe) isoleucine (Ile), threonine (Thr), leucine (Leo), tryptophan (Trp) and valine (Val) and non-essential amino acids such as alanine (Ala), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), glycine (Gly), rhines (Serum) and tyrosine (Tyr). Mineral concentrations can vary between 1.0 to 8.0 g/100 g DM, and significant macroelements (K, P, Mg, Ca, S, and Na) are found. Potassium is the most significant member of this group. There have also been some intriguing micro-elements discovered, including Fe, Mn, Zn, Cu, B, and Se. The amount of minerals a person consumes is crucial to their health. Vitamins E and C are also present in chestnut fruits (Delgado et al., 2016).
Chestnuts are used to make chestnut flour after being peeled, picked manually or mechanically to remove any faded nuts, and subsequently crushed with a hammer or stone mill. This process takes many hours. Before usage and/or sale, chestnut flour (CF) can be kept for a number of months at room temperature or even for a number of years at 40°C. The flour's biochemical makeup is akin to that of other cereals. CF's major ingredient is starch, but it also contains proteins, lipids, minerals, and vitamins (B1, E, and C), all of which make it a great alternative for those who follow a diet free from gluten. The baking industry has seen a growth in the commercial utilization of CF, particularly in European nations. Cakes, biscuits, pasta, milky pudding items, bread, morning cereals, soups, and sauces could be made using chestnut flour (Mete & Dulger Altiner, 2017). Chestnut flour contains a lot of protein, a lot of sugar (20-32%), a lot of carbohydrates (50-60%), a lot of dietary fiber (4-10%), amino acids that are essential (4-7%), and very little fat (2-4%). In addition, it is abundant in potassium, magnesium, and phosphorus as well as vitamins B, C, and E (Sacchetti et al., 2004).
Chestnut flour is reported to have the following composition: 10.79% moisture, 47.80% starch, 21.51% sugar, 9.50% dietary fiber, 3.80% fat, 4.61% protein, and 1.99% ash in a study for the creation of gluten-free bread (Demirkesen et al., 2010).

ROSEHIP AND ROSEHIP POWDER

The rose plant's pseudo fruit is known as a rose hip (RH). According to Chubasik et al. (2008), various species of rosehips, particularly *Rosa canina* L., are regarded as significant sources of both polyphenols and vitamin C. The fact that RH's chemical composition varies based on the cultivar, growing area, climate, maturity, growing practice, and preservation circumstances is an intriguing aspect. Significant changes in RH's organic acids, phenolics, sugars, water-soluble vitamins, and minerals have been noted by numerous researchers (Ercisli, 2007). The presence of phenolics in Rosaceae fruits may in part be responsible for their physiological properties. Phenolics have a wide range of biochemical activity, some of which are anti-mutagenic, anti-carcinogenic, and antioxidant. The high ascorbic acid content of Rosaceae fruits might additionally have a role in their physiological actions. Numerous biochemical processes, such as antioxidant and anti-carcinogenic characteristics, are carried out by ascorbic acid. According to studies (Gao et al., 2000; Ercisli, 2007), the ascorbic acid content of RH ranged from 140 to 1100 mg/100 ml, with *Rosa canina* having the highest concentration at 880 mg/100 ml.
RH also includes pectin, carotenoids, tannins, tocopherol, bioflavonoids, sugars, organic acids, amino acids, and essential oils in addition to vitamin C. *Rosa canina* RH has a nitrogen level of 0.98%. Potassium and phosphorus values are 4860 ppm and 5467 ppm, respectively. RH has calcium and magnesium values of 2867 ppm and 1254 ppm,

respectively, while quantities of iron, copper, manganese, and zinc are 27, 56 and 30 ppm.

Vital fatty acids, which are long-chain, polyunsaturated fatty acids, are another vital component of rosehips. Linoleic and -linolenic acids are important fatty acids that control a variety of physiological processes, including blood viscosity, blood pressure, neuronal function, membrane fluidity, immunological, inflammatory, and many others. *Rosa canina* has 1.78% total fat. *Rosa canina* includes the following main fatty acids in the following proportions: lauric acid (4.88%), palmitic acid (16.4%), linoleic acid (16.0%), linolenic acid (40.5%), and nonadecylic acid (4.74%) (Ercisli, 2007).

Another vital component of rosehips that ought to be mentioned is the galactolipid. According to research, this bioactive molecule exhibits anticancer and anti-inflammatory properties with no reported toxicity (Larsen et al., 2003). More studies and investigations are necessary to properly grasp the potential benefits of *Rosa canina* for human health, despite the fact that the data in support of its use is quite encouraging.

Ascorbic acid, or vitamin C, is abundant in rosehips, which contain 426 milligrams of it in every 100 grams of fruit. According to the Institute of Medicine's 2000 recommendations, men should consume 90 mg of vitamin C daily, while women should consume 75 mg. RH also has a high lycopene content, with 6.8 mg per 100 g of wild RH.

THE HEALTH IMPACT OF USING CHESTNUT FLOUR AND ROSEHIP POWDER AS A FUNCTIONAL FOOD INGREDIENT

Chestnuts as a source of gluten free flour for celiac disease

One of the best-known use of gluten-free flours, such as chestnut flour, is for products that do not contain gluten and that are aimed for consumers with Celiac Disease. Celiac disease (gluten enteropathy), which is a lifelong intolerance to gluten from wheat and other grains that contain proteins comparable to gluten, for example barley, rye, and oats, is recognized as a proximal illness of the small intestine. Instead of using the flour from these cereals, products that use other fruit and vegetable flours should be developed in order to aid in the treatment of this illness through nutrition. People with celiac disease must adhere to a rigorous gluten-free diet that is frequently imbalanced and deficient in nutrients (Kupper, 2005; Chand & Mihas, 2006). Because gluten-free goods are likely to have lower nutritional content than their gluten-rich equivalents, consumers who are sensitive to gluten are searching for high-quality foods (Rocchetti et al., 2019).

The demand for gluten-free goods has rapidly increased in recent years, but celiac patients still need access to fresh, higher-quality goods. Making gluten-free products is difficult because gluten affects a number of crucial structural, rheological, and organoleptic characteristics of bread, snacks, and pasta. There isn't a direct alternative to gluten at the moment. Instead, gluten-free foods are made using a mixture of unfortified refined grain flour, a few hydrocolloids, and proteins. Alternative flours can be used to create acceptable gluten-free products while also enhancing their nutritional value and glycemic index (do Nascimento et al., 2014).

A lot of studies (Demirkesen et al., 2010; Aguilar et al., 2016; Koca et al., 2018; Torra et al., 2021) investigated the utilization and addition of chestnut flour to the composition of certain food products such as biscuits, cookies, bread, cakes, noodles, pasta, muffins, etc., and showed that the products that were developed, are a great alternative for traditional products, that can be consumed by celiac patients.

Chestnuts as a source of dietary fibers

A key ingredient in chestnuts is fiber. On a dry matter (DM) basis, neutral detergent fiber (NDF) makes up 2.7 to 28.9% of the fiber fractions, while acid detergent fiber (ADF) ranges from 0.5 to 4.5%, whereas acid detergent lignin (ADL) merely makes up 0.02 to 1.3% and cellulose makes up 0.5 to 3.6% (DeVries et al., 1999).

Dietary fibers are associated with positive health effects, including “stimulation of *Bifidobacterium* and *Lactobacillus* in the intestine, decrease in cholesterol levels, reduction of the risk of cardiovascular diseases, positive regulation of insulin response, increase in anticancer mechanisms and positive effects on the metabolism of blood lipids" (Prosky, 2000).

The process of fermentation of dietary fiber results in the production of short-chain fatty acids. These substances are crucial for ensuring the preservation of colonic integrity and metabolism. Additionally, according to Cook & Sellin (1998), they can be used as therapeutic agents to treat conditions like colitis, antibiotic-associated diarrhea, and colon cancer.

Chestnuts as a source of natural antioxidants

Chestnuts include vitamins such as vitamin E and C. Unsaturated fatty acids are protected against oxidation by vitamin E. From a nutritional standpoint, vitamin E has demonstrated a number of positive effects for human health, including reducing the negative effects of inflammatory diseases (such as rheumatoid arthritis or hepatitis) (Venkatraman & Chu, 1999), boosting the immune system and lowering the risk of cancer (Lee & Man-Fan, 2000), as well as a possible contribution to lowering the viral load in HIV-infected patients (Allard et al., 1998).

It is believed that vitamin C, the most significant hydrophilic antioxidant, is essential for protecting against illnesses and the degenerative processes brought on by oxidative stress (Retsky et al., 1993). Other organic acids, including citric, malic, quinic, fumaric, and oxalic acids, have also been found in chestnut fruits in addition to ascorbic acid.

It was found that several organic acids had high ranges, presumably as a result of the various extraction techniques used.

Due to their antioxidant action, organic acids may have a preventive impact against a variety of diseases (Carocho et al., 2013).

Inflammatory illnesses, ischemic diseases, cancer, hemochromatosis, emphysema, gastric ulcers, hypertension and preeclampsia, neurological conditions, alcoholism, smoking-related diseases, and others are among the human diseases that can be prevented by the antioxidants found in natural products (Uttara et al., 2009).

Health effect of rosehips consumption

Galactolipid in rosehips gives a unique anti-inflammatory effect. To maximize the preservation of phytochemicals, a standardized rosehip powder was developed. This powder has shown clinical advantages in illnesses like osteoarthritis, rheumatoid arthritis, and inflammatory bowel disease in addition to antioxidant and anti-inflammatory action.

Rosehips are high in vitamin C, as was previously mentioned. Vitamin C, also referred to as L-ascorbic acid, is an essential water-soluble vitamin. Humans are unable to produce vitamin C internally, unlike the majority of animals (Li & Schellhorn, 2007). Numerous biological processes depend on vitamin C, including the formation of collagen, L-carnitine, and norepinephrine. Alpha-tocopherol (vitamin E) and vitamin C have both been found to replenish other antioxidants in the body, making them both significant physiological antioxidants (Fan et al., 2014).

Vitamin C can modify the immune system's response (Carr & Frei, 1999; Jacob & Sotoudeh, 2002), restrict the development of carcinogens such nitrosamines in vivo, and possibly lessen oxidative damage (antioxidant function) that might cause cancer (Li & Schellhorn, 2007). According to the majority of case-control studies (Carr & Frei, 1999; Jacob & Sotoudeh, 2002), there is a negative correlation between dietary vitamin C intake and malignancies that affect the breast, lung, colon or rectum, stomach, oral cavity, larynx or throat, and esophagus. However, there is conflicting information regarding how much dietary vitamin C consumption affects cancer risk. Additionally, the majority of research trials indicate that taking extra vitamin C by itself or in combination with other nutrients has little advantage in terms of preventing cancer (Fan et al., 2014).

Additionally, cancer patients' plasma levels of vitamin C are lower than those of controls (Carr & Frei, 1999).

High-dose intravenous vitamin C therapy is frequently used to treat cancer. According to research done by Casciari et al. (2001), low dosages of ascorbate were cytoprotective but high levels enhanced cancer cell death. Padayatty et al. (2006) demonstrated that intravenous approaches produced plasma concentrations that were approximately 25 times greater than oral vitamin C therapy.

Rosehip aids in reducing the signs and symptoms of rheumatoid arthritis, osteoarthritis, and other conditions. The most prevalent type of arthritis is osteoarthritis. It is a long-term disorder where cartilage deteriorates. As a result of the bones rubbing against one another, stiffness, discomfort, and decreased joint motion result. Recent research has revealed that inflammatory mediators, such as prostaglandins, chemokines, and cytokines, are crucial for the development and maintenance of osteoarthritis (Berenbaum, 2013).

Numerous investigations have been carried out to assess the *in vitro* antioxidant capacities of rosehips and their derivatives. A 50% ethanol crude extract of RHP and its phenolic, ascorbic, and lipophilic fractions were studied by Gao et al. in 2000. The lipid peroxidation (2,2'-azobis (2,4-dimethylvaleronitrile: AMVN) and inhibition (2,2'-Azobis(2-amidino-propane) dihydrochloride: AAPH) tests) and Trolox-equivalent antioxidant capacity (TEAC) assays all revealed high antioxidant activity. According to the relationship between total antioxidant capacity and antioxidant concentration, the phenolic fraction significantly contributed to the antioxidant activity, but the lipophilic component was the most efficient.

In a 2010 study, Egea et al. evaluated the hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2) abatement and total antioxidant capacity (TEAC) of *Rosa canina* RH and five other fruits. The fruits' total phenolic, ascorbic acid, and carotenoid contents were also examined. *Rosa canina* had a significantly higher phenolic and carotenoid content than the other fruits examined. *Rosa canina* RH had a high ascorbic acid concentration, which was indicative of its increased efficacy in the TEAC experiment and against H_2O_2 species. Just phenolics and carotenoids exhibited a minimal connection with the TEAC assay after a collection assessment between every antioxidant and every phytonutrient (Egea et al., 2010). The main ingredient in *Rosa canina*'s seeds, tiliroside, has been shown to have anti-obesity and anti-diabetic properties by promoting the oxidation of fatty acids in the skeletal muscle and liver (Ninomiya et al., 2007; Nagamoto et al., 2013).

CURRENT TRENDS IN EXPLOITING THE FUNCTIONAL POTENTIAL OF CHESTNUT FLOUR AND ROSEHIP POWDER

Studies concerning the utilization of chestnut flour (CF) as a replacement or in addition to traditional raw materials have shown that using CF not only makes the food product more acceptable from a taste point of view, but also fortifies it, and gives it functional properties. In a study (Inkaya et al., 2009) on the functional properties of CF and potential utilization in low-fat cookies, showed that CF is a material that is suitable for cookie production, that depending on the type of CF used, either had no deteriorative effect, or had an improving effect on various properties of the cookie. Dall'Asta et al. (2013) studied the effects of CF supplementation on the physicochemical properties of bread. The results of the study showed that, depending on the content of CF they were formulated with, breads could have a higher antioxidant capacity. Also, compared to regular wheat bread, the breads containing chestnut flour showed a higher amount of volatiles. Bread made with 20 g/100 g of CF showed a heterogenous crumb structure, a less dark color, lower hardness, resulting in a product that would meet consumer satisfaction (Dall'Asta et al., 2013).

Studies on the effect of chestnut flour supplementation on the physicochemical properties and oxidative stability of gluten-free biscuits during storage (Paciulli et al., 2016) were conducted on two lots of biscuits using different flour mixtures. Results indicated that increasing the percentage of CF lead to a color improvement, but excessive amounts of CF, led to a product that was too hard texture-wise. The stability of the color obtained by using CF was confirmed during shelf life, and so was the oxidative stability. The study also made with CF. New pasta products were developed by incorporating chestnut flour and bee pollen, with the intention of creating fortified pasta. The conclusion was that CF represents a promising ingredient for the development of functional, fresh and dried pasta formulas, because of the results. The pasta that was enriched with CF and bee pollen presented a higher amount of fiber, due to the partial replacement of wheat flour with CF. The fat content of the enriched pasta was also notably higher compared to the regular, wheat flour pasta, and, moreover, it was shown to have higher quality because of the higher percentage of unsaturated fatty acids in CF. The enriched pasta also showed a higher protein amount, with high quality proteins and essential amino acids (Brochard et al., 2021).

Bhatt & Jatav (2017) used water chestnut flour to develop and evaluate extruded puss products that were ready to eat. The final product with a content of 10% chestnut flour had an increased amount of essential minerals, starch and protein, compared to the traditional maize, rice flour extruded puff product. The developed product resulted in higher quality, having an overall high acceptability, good sensory qualities, and more nutritional elements. What was interesting in this study was the fact that the product that was developed, was said to be a good nutritional ready-to-eat food for low income groups in developing countries, and also for people with a higher interest in health. This aspect is very important nowadays, when all industries, including the food industry, are searching for ways to make products that can be available for all consumers, no matter their social status or place of living.

An investigation by Dulger & Mete (2020), showed the effect of chestnut flour addition on the nutritional and quality properties of noodles. As in previously mentioned studies, it was concluded that the higher the percentage of CF, the higher the total ash content and dietary fiber was, while the caloric value decreased, compared to the control sample. Thus, it is underlined that chestnut flour containing products can be used in diets, and can also be beneficial for health.

From a sensory point of view, the noodles containing chestnut flour were shown to be acceptable.

Another study shows that chestnut flour can also be used in meat products, such as sausages. Sirini et al. (2020), tested the effects of chestnut flour and probiotic microorganisms on the functionality of dry-cured meat sausages. The results of the study showed that incorporating CF and probiotics in traditional meat products could be a good alternative for adding value to said product. The improvements provided were tendencies to reproduce greater amounts of lactic acid, reducing the presence of residual nitrite, increasing dietary fiber and polyphenols. The ingredients that were incorporated, did not produce significant organoleptic changes, and the final product was acceptable in consumer's preferences.

The suitability of using Rosehip powder as a natural substitute for synthetic ascorbic acid in the bread-making process was examined in a study by Vartolomei & Turtoi (2021). By reducing the moisture, protein, and wet gluten content, raising the ash, fiber, and carbohydrate content, and adding vitamin C at levels corresponding to the amount of rosehip powder used, the replacement of wheat flour with rosehip powder altered the make-up of mixture flours. It also identified variations in bread physico-chemical properties and dough farinographic qualities.

The higher fiber content of all mixed flours led to greater water absorption as compared to the control specimen and flour improved with ascorbic acid.

The rosehip powder's vitamin C content, synthetic ascorbic acid, and the high fiber content of the combination flours all had an impact on the dough growth time, dough stability, and softening degree changes, which were all statistically significant. Furthermore, the inclusion of rosehip powder had a favorable impact on the farinographic quality number. In comparison to the control bread, the bread made from wheat flour and rosehip powder showed a positive evolution of physicochemical properties, including a noticeable increase in height, volume, specific volume, moisture, acidity, and porosity as well as a slight decrease in elasticity. These findings suggest that rosehip powder could take the role of manufactured ascorbic acid while baking bread (Vartolomei & Turtoi, 2021).

CONCLUSIONS

The population of our planet is rising, while the resources are decreasing. In order to feed the present and future generations, a new approach to human nutrition is needed. The development of novel food products that have added value in terms of nutritional and functional properties is the focus of the research in the field of food. The new generation of products should be in accordance with the environmental directives, sustainable, should do no harm, and should also ensure equitability of the market. The novelty products need to meet consumer needs and expectations while making sure that everyone, no matter the social status and the place they live, has access to said products. For novelty products, new materials, even unconventional ones, are needed. There are still plenty of resources that could be put to use in the creation of better products but have either not been exploited at all or not enough. The chestnut is one of these resources. A sustainable resource that has not been sufficiently exploited, could be the answer to many issues the planet faces: from food and nutrition, to waste reduction. The knowledge gathered from all the references cited below is proof that food and nutrition-wise, chestnuts and chestnut flour is an ingredient fit for the future. Further studies on the matter must be conducted, in order to fill the gaps in today's knowledge, and to explore thoroughly the effects this novel material might have, both on the food industry, and other industries as well.

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A PRELIMINARY INVESTIGATION INTO THE ENHANCEMENT OF CHEESE WITH GRAPE SKIN POWDER

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Abstract

Improving management and making the food industry more sustainable requires a focus on reducing waste and finding uses for by-products. In the case of grapes, by-products account for approximately 20%. It is worth noting that these by-products contain beneficial phenolic compounds that have anti-allergenic, antibacterial, anti-carcinogenic, anti-inflammatory, antioxidant, and cardioprotective effects. For these reasons, the food industry sector must turn its attention to them and use them as functional ingredients. In this study, grape skin powder (GSP) was added to cheese to increase its antioxidant and bioactive compounds content. The enriched cheese contained significantly higher levels of total phenolic content (TPC) and antioxidant activity compared to the control sample. Adding 2% grape skin powder resulted in an increase of 1.927 mg GAE/g DW (mg gallic acid equivalents (GAE) per gram of dry weight (DW)) of TPC in cheese formulation. The GSP-supplemented cheese also showed greater antioxidant activity than the control. This study demonstrates that grape by-products can effectively transfer beneficial compounds to cheese.

Key words: *by-products, cheese, food, quality.*

INTRODUCTION

One of the most pressing issues that the food industries are grappling with is the alarming amount of waste that they generate (Comunian, et al., 2021). This waste is largely made up of food by-products that are discarded without any regard for their potential value. This problem is not just an isolated issue but is, in fact, a major global challenge that demands our immediate attention. The sheer scale of this problem is staggering, and it represents a inefficient use of our precious natural resources. It is high time that we take this issue seriously and work towards finding sustainable solutions to address this problem (Bedoić et al., 2019).

Agricultural and food by-products can play a role in the production of animal feed and the development of sustainable functional foods (Bedoić et al., 2019).

To achieve this, we must efficiently utilize waste as raw materials and promote strategies that are rooted in green and sustainable technologies. In doing so, we can ensure that we leverage the full potential of these valuable resources while minimizing their environmental impact (Gustavsson et al., 2011).

Grapes are widely regarded as one of the most extensively cultivated fruits across the globe. Specifically, in the continent of Europe, a staggering 3.5 million hectares of land are solely dedicated to the cultivation of this succulent fruit. As a result of this effort, an impressive 27 million tons of grapes are produced annually, highlighting the sheer scale and importance of grape cultivation in this region (FAO, 2019). It has been estimated that each year, some 14.5 million tons of grape by-products are produced. This quantity of waste is primarily composed of grape pomace, which makes up a significant portion (50-65%) of the total by-product volume (Teixeira et al., 2014). Indeed, grape skin is the main constituent of this pomace, underscoring its importance as a source of valuable nutrients and bioactive compounds (Aliaño-González et al., 2022).

These by-products are of utmost importance from an environmental perspective, as they can be utilized as sources of nutrients and various bioactive substances for internal nutrition and feeding (Câmara et al., 2020).

Additionally, when it comes to mineral composition, grape pomace is abundant in Ca, P, Mg, K, and Fe content (Shrikhande, 2000; Galanakis, 2017). Therefore, it is crucial to utilize these by-products to their fullest potential, not only for their nutritional value but also for their positive impact on the environment (Hassan et al., 2020).

The addition of grape by-products to various food items will undoubtedly result in the creation of functional food products, thereby introducing natural functional food ingredients such as dietary fibers and polyphenols into commonly consumed foods. Such practices not only benefit our health but also have a positive impact on the environment.

A significant development in the food industry has been the utilization of grape by-products in various sectors such as the beverage, bakery, and dairy industries (Gaglio et al., 2021). This innovative approach has enabled manufacturers to maximize the potential of grapes, not just for their fruit or juice but also for their by-products. As a result, grape by-products have become an essential ingredient in the food industry, providing a new dimension of flavour and nutritional benefits to a wide range of products (Chouchouli, 2013).

To sum up, after conducting several studies, it has been discovered that grape and wine by-products hold significant importance as a rich source of functional compounds that can be utilized in the production of various dairy products. These dairy products may include fermented milks, yogurts, cheeses, or ice-creams (Dos Santos et al., 2017). It is noteworthy to mention that these by-products have been found to contain a plethora of beneficial nutrients that can enhance the nutritional value of the final products (Pavlou, 2021).

Furthermore, it has been suggested that incorporating grape by-products into dairy products could be a viable method for enhancing their shelf life, as demonstrated in previous research (Tseng et al., 2013). This is due to the fact that grape by-products are known to contain high levels of phenolic compounds and antioxidants, which are not typically found in dairy products (Albu et al., 2018; Kandylis et al., 2021).

Interestingly, cheese in particular may benefit from the addition of grape by-products, as they have been shown to significantly increase the total phenolic content and radical scavenging activity of this popular dairy product (Gaglio et al., 2021). Additionally, studies have evaluated the impact of grape by-products on the microbiological aspects of dairy production, with promising results reported in numerous cases (Barbaccia et al., 2022). Overall, it appears that grape by-products have great potential as a functional ingredient for enhancing the quality and longevity of dairy products.

Furthermore, the addition of grape by-products can reduce the fat content of cheese and increase protein levels since GPP (grape pomace powder) are low in lipid components, including them in cheese led to a reduction in fat while simultaneously increasing protein content (Gaglio et al., 2021). Grape by-products can serve as coagulant in the production of tofu and may change its textural parameters, and even its colour (Zeppa et al., 2021). Therefore, incorporating these by-products into dairy formulations can be a wise and healthy choice, which can also contribute to the sustainability of the food industry (Albu et al., 2018; Baron et al., 2021).

For this reason, the main objective of the present study was to investigate the potential benefits of grape skin powder in terms of providing natural antioxidants and other lipophilic bioactive compounds to the cheese composition of the assorted cheese products. In order to achieve this goal, a thorough examination was conducted on the efficacy of grape skin powder as a viable source of these crucial components. The findings of this research have significant implications for the cheese industry, as the use of natural, plant-based ingredients can enhance the nutritional value and overall quality of the cheese products. Therefore, this study aims to provide valuable insights into the use of grape skin powder as a functional ingredient in the cheese-making process.

MATERIALS AND METHODS

30 kg of Fetească Neagră grapes from USV-Iasi's farm were washed, dried and hand-separated (Figure 1 a). The skin was lab-dried at 40ºC with 1 m/s air speed for the powder preparation (Figure 1 b, c).

a. Pulp removal b. Skin dry c. Powder

Figure 1 Obtaining the powder from the grape skin

The Rediu Iași Research Station, under the University of Life Sciences, supplied 300 L of cow's milk from their 55 Fleckvieh/Simmental cattle.

Extraction of bioactive compounds from grape skin powder (GSP). A quantity of 1 g of dried peels grape powder was utilized for the ultrasound-assisted extraction along with 9 mL of the solvent (80% ethanol) and 1 ml of 1% citric acid. The extractions took place at 40°C for 45 minutes using a sonication water bath (MRC Scientific 193 Instruments, Holon, Israel). In order to obtain extracts rich in anthocyanins, the extraction process was repeated three times. The supernatants were then collected and centrifuged for 10 minutes at 6000 rpm and 4°C. The resultant supernatants were concentrated to dryness at temperature of 40 °C, under reduced pressure (AVC 2-18, Christ, Shropshire, UK), and the obtained extract was dissolved in the extraction solvent and then used for the phytochemical characterization.

Determination of the Total Anthocyanins Content (TAC). A modified pH differential technique was utilized to determine the total monomeric anthocyanins content (TAC) (Lee et al., 2005). Prior to determination, the samples were diluted (1:10) with the extraction solvent. The absorbance of the diluted extracts was then measured at two different wavelengths, 520 nm, and 700 nm (UV-VIS spectrophotometer Libra S22, Biochrom, UK), using 200 µL of vegetable extract and 800 µL of two different buffers solutions (0.025 M potassium chloride buffer at pH 1.0 and 0.4 M sodium acetate buffer at pH 4.5). Results were given in milligrams of cyanidin-3-glucoside (C3G) per gram of dry weight (DW) (mg C3G/g DW).

Determination of the Total Phenolic Compounds (TPC). The total phenolic compounds content (TPC) in the extract was determined by the Folin-Ciocâlteau method (Sant'Anna et al. 2012) using Gallic acid as standard. Briefly, 1 mL of Folin-Ciocâlteu reagent and 15.8 mL distilled water were added to 200 µL of extract, to a final volume of 17 mL. 3 mL of 20% Na_2CO_3 was added after 10 minutes, and the mixture was kept at 25°C in a dark place for 60 minutes. The absorbance of the reaction mixture was measured at a wavelength of 765 nm by UV-VIS spectrophotometer with data analysis software (Libra S22, Biochrom, UK) and the results were expressed as mg gallic acid equivalents (GAE) per gram of dry weight (DW) (mg GAE/g DW).

Determination of the Total Flavonoids Compounds (TFC). The aluminium chloride colourimetric method was used for the determination of the total flavonoids compounds (Dewanto et al., 2002). For TFC content the assay involved the mixing of 0.25 mL of extract sample with 1.25 mL of distilled water and 0.075 mL of 5% sodium nitrite solution. A volume of 0.150 mL of 10% aluminium chloride solution was added and allowed to react for 6 min at room temperature after the initial 5 min of reaction. In addition, 0.750 mL of distilled water and 0.5 mL of 1M sodium hydroxide were added, and the mixtures' absorbance was then measured instantly at 510 nm by UV-VIS spectrophotometer with data analysis software (Libra S22, Biochrom, UK). The TFC content was expressed in mg catechin equivalents (CE) per g dry weight (mg CE/g DW), based on the catechin standard curve.

Determination of antioxidant activity (DPPH). The antioxidant activity was determined by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay. The capacity to inhibit the DPPH radical derived from the fact that the purple colour of DPPH turns yellow when the full quantity of free radicals is blocked by the antioxidants. The DPPH scavenging activity was measured by using the method described by Turturică et al. (2015). Briefly, a mixture was obtained by adding 3.9 mL of 0.1 M DPPH solution and 200 μL extract. The mixture was stored at 25°C for 90 minutes in a dark area. The mixture absorbance was determined at a 515 nm wavelength by UV-VIS spectrophotometer with data analysis software (Libra S22, Biochrom, UK). Also, a control was made by mixing 3.9 mL DPPH solution 0.1 M and 200 μL methanol. The absorbance mixture was then measured.

The results obtained were expressed as mM Trolox/ g DW.

The DPPH Inhibition (%) was calculated using the following formula:

$$\% \text{ Inhibition} = (A_{blank} - A_{sample}/A_{blank}) \times 100,$$

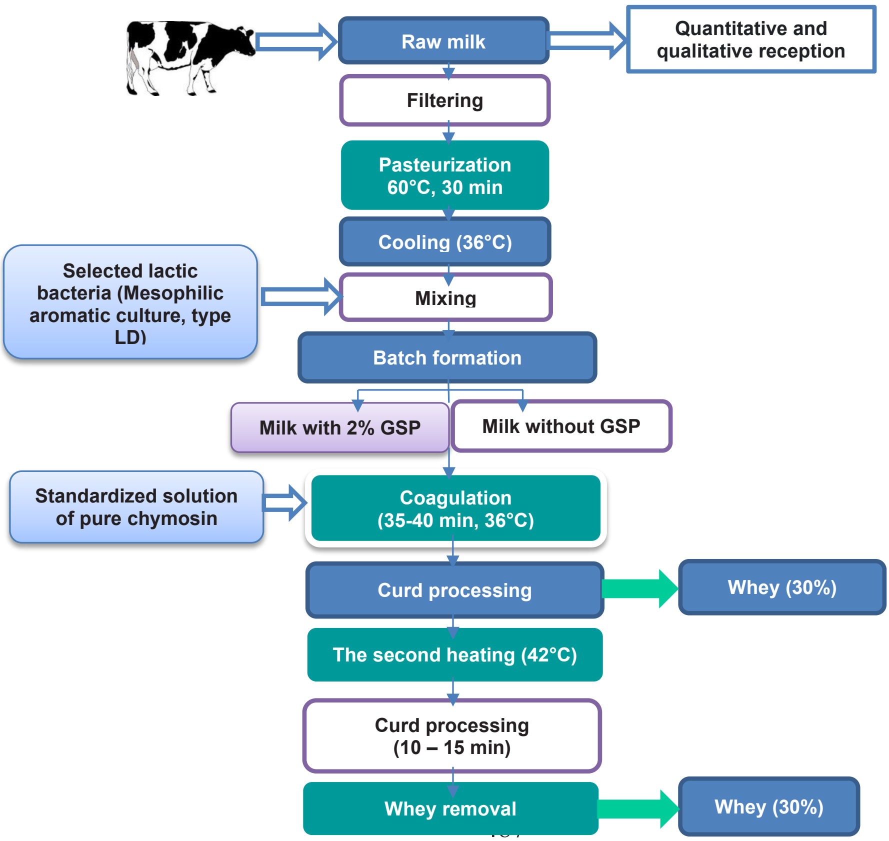
A_{blank} - absorbance of the control sample;

A_{sample} - absorbance of the sample.

Cheese making. 300 L of milk were taken from the farm's storage tank and delivered to the University of Life Sciences for processing. The milk was kept at 5°C in a storage tank. Five samples were taken from the reservoir in sterile containers weighing 300 ml each. The samples were transported in a separate box with ice packs and refrigerated at 4°C for 24 hours.

The milk was homogenized and analysed in the laboratory with five replications per trait/method. Physicochemical parameters of milk samples were analysed using AOAC, 1990 methods to determine moisture, solid non-fat, fat, protein, ash and pH.

In Figure 2 is the technological flow of cheese manufacturing (Caciotta cheese).



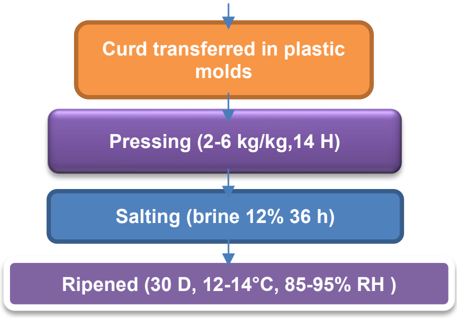


Figure 2. Technological flow of cheese manufacturing

In this case, the milk used in the technological process was not normalized (full-fat milk was used). The milk was pasteurized at 60°C for 30 minutes, after which it was cooled to 36°C degrees, at which point selected lactic bacteria were added. After homogenization, the two batches (CC and CGSP) were created. Coagulation, coagulum processing and molding (putting the coagulum into forms) was done separately for each batch.

Regarding the part of the qualitative analysis carried out to establish the qualitative parameters of both the product with the addition (Cheese with grapes skin powder - CGSP) and the control product (CC), sensory, physical (pH, colour and texture) and chemical analysis were performed (water, dry matter, F/DM (fat reported on dry matter content), protein, ash and salt content).

Sensory session. The samples were evaluated descriptively through the use of standard terminology and references. Two separate samples were examined during the sensory session for each of the qualities, with each sample being divided into three replicates for each of the assessors. Muir et al., (1996), describe an experimental strategy utilized to remove carryover and order of tasting bias is detailed in depth. 16 characteristics for the tested cheeses were chosen and carefully defined for profiling using the QDA (Qualitative Data Analysis) approach (Table 1).

Table 1. Attribute list used in QDA analysis

Descriptor	Characteristics
Colour	visual intensity measurement
Creamy odour	market-cream odour (30%)
Acid odour	A characteristic flavour of fermented milk products like yoghurt
Buttery	flavour connected to butter
Flowers/fruits	the fragrant fusion of several fruity flavours, including red berries, juicy apples, and pineapple. Additionally, it might contain the aromas connected to sweetened cultured dairy products such fruit yoghurts.
Salty	sodium chloride's characteristic basic flavour when diluted in water (0.2%)
Sweet	basic taste experience that sucrose often has
Acid	fermented milk products' flavour
Bitter	flavour, basic caffeine taste in water (0.5%)
Aftertaste	aftertaste that persisted after the sample was taken away
Hardness*	the amount of force required by the jaws to tear the sample in half
Chewiness	length and frequency of chewing the substance before swallowing
Rubbery	the sample's capacity to reshape itself after pulling
Dryness (moisture)	moisture in the sample, mouthfeel after four or five chews
Grainy	the sample's capacity to splinter into parts
Overall quality	general impression based on what people like and dislike

These characteristics included colour, scent (creamy, acid, buttery, fruits), taste (salty, acid, sweet, bitter, aftertaste), and texture (hardness, chewiness, rubbery, dryness, grainy). Continuous unstructured graphical scales were used to rate the attribute intensities. The scales

were 10 cm long with verbal anchors at either end, with the left side representing the attribute's lowest intensity (value 0) and the right side representing its highest intensity (value 10). The
same type of scale as described above, which is anchored at both ends, was used to evaluate the overall quality of cheeses: unlinking (0)-extremely linking (10).

Physical parameters. pH was determined by potentiometric analysis, with the electrode being inserted directly into the cheese wheel.

The colour characteristics were measured using the MINOLTA Chroma Meter model CR-410 (Konica Minolta, Osaka, Japan) with a CIE Lab scale. The Chroma parameters were read using A* (red (> 0) to green (< 0) colour), b* (yellow (> 0) to blue (<0) colour), and L* (black: L* = 0 and white: L* = 100). The results were expressed in triplicate, following equipment calibration against a white plate. The colour was described by hue angle (Hue angle = arctan(b*/a*) for quadrant I (+a*, +b*), where 0° or 360° indicated red colour, 90° indicated yellow colour, 180° indicated green colour, and 270° indicated blue colour. The purity or saturation of colour, also known as chroma, was also determined (Chroma = $\sqrt{(\mathbf{a}^*)^2 + (\mathbf{b}^*)^2}$)(Dag et al., 2017).

The statistical analysis was performed using the SPSS program (ver. 19), which has a multi-function utility with regard to the experimental design. comparisons were performed using single factor ANOVA in according with Pallant, (2016).

Chemical parameters. The quantification of the fat content was accomplished through the Soxhlet extraction technique (Wojciechowski et al., 2016), a widely recognized and accepted analytical technique. In tandem, the protein content was assessed using the Kjeldahl method, another established and precise method for measuring this nutritional component (Barbano & Clark, 1990; Lynch & Barbano, 2006). Additionally, the determination of water, dry matter and F/DM content were conducted in strict accordance with the AOAC's 2012 guidelines, a well-respected and rigorously researched set of protocols. Titration with $AgNO_3$ (International Standard FIL-IDF 88/A: 1988) was used to determine the concentration of sodium chloride (salt content).

RESULTS AND DISCUSSIONS

Characterization of the GSP extract. In the current study, the extraction process was performed using the ultrasound-assisted extraction method, with ethanol 80% and citric acid (1%) as a solvent. The biologically active compounds content and antioxidant activity of the GSP extract were evaluated and the results are presented in Table 2.

Table 2. Phytochemical content and antioxidant activity of the GSP extract

Sample	TAC[1] (mg C3G/g DW)	TFC[2] (mg CE/g DW)	TPC[3] (mg GAE/g DW)	DPPH[4] (mM Trolox /g DW)	Inhibition %
GSP[5]	1.242±0.019	10.175±0.128	22.357±0.535	20.591±0.093	78.587 ±0.355

[1] - Total Anthocyanins Content
[2] - Total Flavonoids Compounds
[3] - Total Phenolic Compounds
[4] - Antioxidant Activity (DPPH)
[5] - Grape Skin Powder

The extract's TAC was 1.242±0.019 mg C3G/g DW, and its TFC and TPC values were 10.175 ± 0.128 mg CE/g DW, and 22.357 ± 0.535 mg GAE/g DW, respectively. The GSP extract's radical-scavenging activity was 20.591 ± 0.093 mM Trolox/g DW, with 78.587 ± 0.355% inhibition of DPPH radical.

Therefore, Serea et al., 2021 reported similar values for anthocyanins (1.36 ± 0.14 mg C3G/g DW), polyphenols (16.51 ± 3.20 mg GAE/g DW), flavonoids contents (10.61 ± 0.82 mg CE/g DW), and for antioxidant activity (15.20 ± 0.70 mM Trolox/g DW) in ultrasound-assisted extracts of *Băbească neagră* red grapes skins using 96% ethanol at 25ºC for 55 minutes.

Additionally, Constantin et al., 2015 extracted the biologically active compounds from *Fetească neagră* red grapes skins using a conventional method (at 28ºC for 2 hours) and reported anthocyanins of 18.54 ± 0.34 mg C3G/g DW, flavonoids of 2.27 ± 0.20 mg CE/g DW, polyphenols of 7.42 ± 0.06 mg GAE/g DW, and antioxidant activity of 4.89 ± 0.02 µM Trolox/g DW (by ABTS assay). Li et al., 2019 utilized the following extraction parameters after model validation: 49% ethanol, at 51°C and 15 minutes; to achieve an antioxidant activity of 41.78 ± 1.13 mg Trolox/g DW, the total phenolic content of 15.24 ± 0.73 mg GAE/g DW), and total anthocyanins content of 346.68 ± 9.10 mg C3G/100 g DW. Similar results for grape skin extract flavonoids were achieved by Ivanova et al., 2010, who reported a value of 6.90±0.42 mg CE/g DW following extraction with 80% ethanol. A lower concentration of TPC of 33.3±0.3 mg GAE/100 g DW skin of red grapes was reported by Negro et al., 2003 by extracting the compounds with 80% ethanol and acetic acid. On the other hand, Manconi et al., 2016 stated a higher amount of phenolic compounds of 126 ± 30 mg GAE/g DW from grape pomace and a lower radical scavenging activity by using a DPPH• assay of 0.91 ± 0.17 mM Trolox/g DW compared with the current results. De Andrade et al., 2021 reported similar findings and found that the skin of *Syrah* grapes cultivar contained 3.25 ± 0.03 mg malvidin-3-O-glucoside/g DW of anthocyanins. According to Mendoza et al., 2013, the concentration of anthocyanins in different grape pomace cultivars ranged between 20.74 ± 0.02 and 70.86 ± 5.71 mg malvidin-3-O-glucoside/100 g DW in a considerably lower amount compared with our results. In contrast, Tan et al., 2020 reported that the anthocyanins content of the grape skin extract via ultrasound-assisted enzymatic extraction was 3.01±0.04 mg C3G/g DW, obtained under an extraction temperature of 50°C, ultrasonic power of 400 W, pectinase dosage of 0.16%, and extraction time of 28 minutes.

However, the phytochemical composition of GSP extracts can vary with different origins of raw materials, cultivar (i.e., genetic and environmental features), agronomic practices, different extraction conditions (type of solvent, pH, temperature), and methods, and the measurements method applied.

Physicochemical parameters of milk samples. Table 3 presents the chemical composition results of the cow's milk samples used as the raw material for cheese production.

Table 3 Chemical composition (%) of raw cow's milk samples (n = 5)

Parameters	Mean	±Sx	Interval	Variance
Water (%)	86.88	0.128	86.40-87.15	0.330
Total solids (%) (TS)	13.12	0.128	12.85-13.60	2.182
Fat (%)	4.00	0.027	3.95-4.10	1.534
Solid-non fat (SNF) (%)	9.12	0.129	8.88-9.59	0.014
Protein (%)	3.32	0.014	3.29-3.36	0.976
Ash (%)	0.50	0.007	0.48-0.52	3.162
pH	6.58	0.009	6.55-6.60	0.292

In terms of its composition, the milk sample had an average water content of 86.88 ± 0.128% and a total solids content of 13.12 ± 0.128%. Water plays a crucial role as the medium in which all other components of milk are dissolved or suspended. The average fat content was 4.00 ± 0.027%, resulting in an average solid-non fat (SNF) content of 9.12±0.129%. The protein level was recorded at an average value of 3.32 ± 0.014%, while the ash content was found to be 0.50 ± 0.007%, with variation limits between 0.48% and 0.52%. These findings were consistent with those reported by Amitot et al., 2002. The pH value of the milk ranged between 6.55 and 6.60, with an average value of 6.58 ± 0.009, which was similar to the results reported by Danthine et al., 2000.

Phytochemical profile of value-added cheese. The value-added Caciotta cheese sample was obtained by enrichment with 2% amounts of grape skin powder. Bioactive compounds content were measured in control and experimental cheese samples to assess the impact of adding GSP to cheese. The obtained Caciotta cheese's phytochemical content and antioxidant activity are shown in Table 4.

Table 4 Phytochemical profile of value-added Caciotta cheese

Sample	TAC, mg C3G/100 g DW	TFC, mg CE/g DW	TPC, mg GAE/g DW	Antioxidant activity, mM Trolox/g DW
CC	-	0.313 ± 0.005^{a}	2.264 ± 0.029^{a}	1.574 ± 0.057^{a}
CGSP (2%)	4.661 ± 0.031	0.517 ± 0.004^{b}	4.191 ± 0.039^{b}	2.425 ± 0.091^{b}

Values with different letters in the same column are significantly different (p<0.05).

Statistically significant differences (p<0.05) were discovered among cheeses. As anticipated, the control cheese (CC) had lower amounts of bioactive compounds than the corresponding CGSP-enriched cheeses.

The anthocyanins were not detected in sample CC, and flavonoid content had a low level (0.313 ± 0.005 mg CE/g DW). The contents of the bioactive compounds were found to increase when GSP (2%) was added to the cheese formulation. Moreover, the sample obtained had considerably higher levels of bioactive compounds in comparison to the control sample, exhibiting functional characteristics (total phenolic content and antioxidant activity). In particular, the addition of 2% (*w/w*) of GSP resulted in an increase of 1.927 mg GAE/g DW of TPC in Caciotta cheese formulation.

Furthermore, the GSP-supplemented cheese sample showed greater antioxidant activity than the control due to the increased concentrations of bioactive compounds in the GSP.

These findings supported the hypothesis that the addition of GSP to dairy products causes an increase in the phytochemical contents especially TPC (Barbaccia et al., 2022; Marchiani et al., 2016a; Marchiani et al., 2016b; Aiello et al. 2020). In order to study the effect of green tea catechins on antioxidant characteristics, Rashidinejad et al., (2016) added green tea extract to full-fat cheeses. As a result, authors noticed a considerable increase in TPC and antioxidant activity at all levels.

Additionally, Costa et al. (2018) showed that Primosale cheese samples enriched with red wine grape pomace (enriched to 50 g/kg) have higher contents of bioactive compounds (polyphenols 6.92 ± 0.38 mg GAE/g DW; flavonoids 1.15 ± 0.24 mg quercetin equivalents/g DW) and significant antioxidant activity (ABTS) (8.44 ± 0.11 mM Trolox/g DW) compared to cheese without grape pomace (control), demonstrating added value.

The literature has reported a wide range of phenolic content for various types of cheese, including up to 11.3 mg GAE/g DW, flavonoids up to 0.9 mg of quercetin equivalent/g DW, and antioxidant activities up to 4.00 mg of Trolox/g DW or 31.14 mM Trolox/g DW (Da Silva et al., 2015; Han et al., 2011; Lucera et al., 2018).

Sensory evaluation of cheese. To evaluate the sensory aspects of the product, we employed the quantitative descriptive analysis (QDA) method, which is commonly used in studies of various products, including cheese (Stone & Sidel 2017; Lawless & Heymann 2010). Table 5 outlines the mean intensity ratings of descriptive attributes and the analysis of variance. Our ANOVA results indicate that there were significant (P < 0.05) differences in the intensity of various attributes, including colour, creamy odour, flavour, salty taste, sweet taste, acid taste, bitter taste, aftertaste, hardness, chewiness, rubbery, dryness, grainy, and overall quality.

Table 5. Quantitative descriptive analysis of CC and CGSP cheeses

Specification	**Type of cheese**	
	CC	**CGSP**
Colour	5.7^{a}	6.13^{b}
Creamy odour	5.9^{a}	4.1^{b}
Acid odour	4.3^{a}	4.7^{a}
Buttery	$6,2^{a}$	$5,1^{b}$
Flouers/fruits	1.3	6.7^{b}
Salty taste	3.7^{a}	3.4^{a}

Sweet taste	4.1^{a}	6.4^{b}
Acid taste	2.7^{a}	1.5^{b}
Bitter taste	1.6^{a}	2.3^{b}
Aftertaste	3.3^{a}	6.9^{b}
Hardness	6.5^{a}	3.9^{b}
Chewiness	6.6^{a}	4.5^{b}
Rubbery	6.5^{a}	5.1^{b}
Dryness (moisture)	3.1^{a}	2.8^{a}
Grainy	5.8^{a}	4.1^{b}
Overall quality	5.7^{a}	6.8^{b}

Letters describe a comparison between each variety of cheese; means marked with the same letters in each row do not show significant differences (ANOVA test, $P < 0.05$).

In Figure 3, it is evident that the inclusion of GSP had a noteworthy impact on the colour of the product. However, this particular attribute was highly valued by consumers, as indicated by the fact that CGSP received a higher score in comparison to CC.

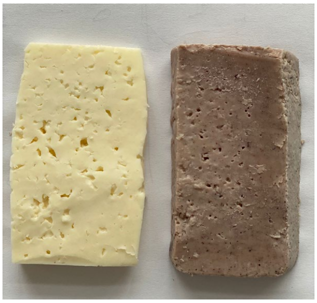


Figure 3. CC and CGSP cheese

It is important to note that this outcome may be attributed to the unique properties of GSP, which contributed to the enhanced colour of the final product. Overall, these findings suggest that the addition of GSP can lead to a favorable sensory experience for consumers, particularly in terms of colour perception (Table 4). These findings serve as a validation of the critical role that visual assessment plays in accurately depicting and valuing food items, as established by Dinnella et al. (2014) study.
When considering the visual aspects, it was observed that the red grape skins from Fetească Neagră contain a significant amount of coloured phenol compounds. These compounds were then released from the grape skins into the cheese, resulting in a unique violet and brown marbling effect that was not present in the reference sample. Similar effects have been observed in other studies that have utilized phenol-based winery by-products in the production of biscuits (Mildner-Szkudlarz et al., 2013; Pasqualone et al., 2014). Additionally, the addition of grape skin powder (GSP) had a substantial impact on the texture of the cheese.
According to the obtained results, it was observed that the inclusion of solid particles within the food matrix led to an increase in the perceived sensation of roughness, while simultaneously causing a significant reduction in the ratings attributed to a number of texture-related characteristics, including hardness, chewiness, rubbery consistency, dryness and graininess. These findings were also reported by Engelen et al. (2005).
Physical parameters. Table 6 details how cheese supplemented with GSP changes in colour. The non-added product (CC) had a distinctively yellow tint.

Table 6. Colour traits and pH of CC and CGSP cheeses

Specification	Type of cheese	
	CC	**CGSP**
L*	68.31±0.04^{a}	53.33±0.09^{b}
a*	-1.22±0.27^{a}	8.31±0.06^{b}
b*	11.59±0.05^{a}	6.46±0.08^{b}
C*	11.66±0.05^{a}	10.53±0.10^{b}
h°	-1.47±0.02^{a}	0.66±0.033^{b}
pH	5.21±0.01^{a}	5.14±0.004^{b}

L* value is the lightness coefficient; b* value indicates the position on the blue (−) to yellow (+) axis; C* (chroma); h° (hue angle). Letters describe a comparison between each variety of cheese; means marked with the same letters in each row do not show significant differences (ANOVA test, $P < 0.05$)

Because of the presence of anthocyanins, the addition of powder to milk entirely altered the product's hue. The introduction of GSP, resulted in a significantly lower brightness (L*) value in the CGSP sample ($P < 0.05$). In the instance of the CGSP product, an increase in the parameter a* was clearly seen as expected ($P < 0.05$). This is a result of the anthocyanins that were added, which are in charge of giving GSP its red hue. Eventually, the parameter b* in the GSP-enriched product significantly decreased ($P < 0.05$). Regarding the results for Chroma, the average was 11.66 ± 0.05 for CC and 10.53 ± 0.10 3 for CGSP. The differences were similarly very significant ($P < 0.05$).

The mean values for the Hue angle parameter were -1.47 ± 0.02 for CC and 0.66 ± 0.033 for CGSP, with a very significant difference ($P < 0.05$) between the two.

In terms of pH value, differences were also found ($P < 0.05$). When GSP was added to cheese, the pH dropped from 5.21 ± 0.01 in the control batch (CC) to 5.14 ± 0.004 in the CGSP batch ($P < 0.05$). Mohamed et al., (2014) also observed a similar pattern in their yoghurt consumption data. Decomposition of the emulsifying salts and/or their interaction with protein during storage likely led to a gradual reduction in pH values across all treatments (Chamber and Daurelles, 2000).

Similar to our research on colour, it has also been carried out on bakery and pastry products. According to Hayta et al. (2014) and Nakov et al. (2020), GPP inclusion caused a significant reduction in brightness (L*) in samples of breeds and cakes.

Demirkol et al. (2018) in a study made on yogurt observed that the L and b* values decreased ($P < 0.05$) but the a* value increased ($P <0.05$).

A decrease in the pH of products that have been supplemented with grape skin powder or grape pomace powder has been demonstrated in several products, not just in dairy products (Shirahigue et al., 2010; Rosales Soto et al., 2012; Frumento et al., 2013).

Chemical parameters. Differences ($P < 0.05$) were found for every parameter in the examination of the chemical quality parameters carried out on the two types of cheese (CC and CGSP), with the exception of fat (Table 6).

Table 6 Chemical composition (%) of CC and CGSP cheeses (n = 5)

Parameters	Type of cheese	Mean	±Sx	Interval	Variance
Water (%)	CC	37.93[a]	0.08	37.63-38.13	0.49
	CGSP	35.97[b]	0.33	34.99-36.82	2.05
Dry matter (%) (DM)	CC	62.07[a]	0.08	61.87-63.37	0.30
	CGSP	64.03[b]	0.33	63.18-65.01	1.15
Fat (%)	CC	33.95[a]	0.09	33.61-34.12	0.59
	CGSP	34.13[a]	0.10	33.86-34.47	0.66
Fat in dry matter (FDM) (%)	CC	57.70[a]	0.16	54.17-55.05	0.66
	CGSP	53.31[b]	0.39	52.33-54.33	1.62
Protein (%)	CC	24.21[a]	0.08	23.98-24.44	0.73
	CGSP	26.06[b]	0.12	24.66-25.41	1.08
Ash (%)	CC	3.87[a]	0.18	4.42	10.12
	CGSP	4.82[b]	0.38	3.85 – 5.60	17.68

Values with different letters in the same column are significantly different ($P < 0.05$).

According to expectations, the addition of powder made from grape skins increased the amount of dry matter; the average value was 64,03% for the product with addition (CGSP) compared to 62,07% for the control product (CC).

However, the improvement of the chemical composition of dairy products to which powder obtained from the skin of grapes or grape pomace powder was added was also reported by Han et al. (2011a, b) who observed that dairy products with grapes and their derivatives introduce to their matrix a large number of phenolic compounds, which have the ability to associate with proteins and carbohydrates. Marchiani et al. (2016a) investigated the addition of red and white Chardonnay and Barbera grape pomaces, both before and after distillation, to Cheddar and Toma-like cheeses. They noted variations in moisture content in the cheeses that were produced as well as between grape pomaces. Arunkumar (2014) also noted a decrease in moisture content, which is explicable given the rise in total solids brought on by the addition of grape pomace flour during cheese manufacture.

CONCLUSIONS

The findings of the study shed light on an interesting fact - grape by-products extracts (GSP) contain a significant amount of total phenolic compounds, total flavonoids, and antioxidant activity that can be effectively transferred to cheeses. This discovery presents a promising avenue for reducing the environmental impact of winemaking across the globe, while also creating new possibilities for the dairy industry to develop innovative dairy products with enhanced functional properties. This is especially relevant today, as consumers are increasingly concerned about the environment and actively seek out healthier food options.

However, it is important to note that while the use of grapes and their derivatives in dairy products offers numerous benefits, it is crucial to pay close attention to their impact on acidity and sensory characteristics. This will ensure that the final product not only boasts functional properties but also meets consumers' expectations in terms of flavour and texture. By carefully balancing these factors, the dairy industry can capitalize on this exciting new opportunity to create products that are both healthy and delicious.

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OVERVIEW ON THE WAYS TO LOSSES REDUCTION AND EFFICIENCY OF FISH PROCESSING

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Abstract

A detailed bibliographic study finds that fish is a very important human food, valued by its balanced content of protein, lipids, and biologically active components. From the up-to-date statistics, it finds out that fish represents an important percentage of human food and protein sources. However, fish, like many other animal food sources, represents a future problem. In the food industry, fish can be sold as such, preserved, or processed in different ways. During fish processing technology, fish by-products are also generated and this represents a problem for the food industry and for the environment also. Transformation of these by-products into different products or their use in other industrial applications can solve the problems and may become a sustainable solution for the industry. This bibliographic study represents the starting point of the food innovation process, by analyzing the actual use of the by-products as medicinal products, artificial pearls, fish skin, isinglass, glue, body oil, manure and guano, silage, soluble, flour, fins, biscuits, macaroni, sausage, ham, liver oil and others and finding new paths of developments in order to achieve the goal.

Key words*: by-products, fish value chain, sustainability, waste management.*

INTRODUCTION

Current fishing practices result in 20 million tonnes of important resources being wasted every year. Even in this situation, vessels must retain on board and bring ashore both target and by-catch species (as required by EU legislation). Therefore, a significant amount of marine biomass of low economic value must be effectively managed to avoid wastage (Antelo et al., 2015).

The fish industry has grown steadily in recent decades and produces massive quantities of by-products. These by-products originate from fish heads, skins, viscera, fins and bones (Marti-Quijal et al., 2020).

Fish is a highly perishable food raw material due to its high moisture and nutrient content. Fish spoilage starts as soon as the fish dies and is the consequence of a series of complex chemical reactions taking place in dead fish, mainly produced by bacteria and enzymes (Subhendu, 2013).

Large quantities of waste, comprising water and solids, are produced in fish processing. The solid waste include 50-70% of the composition of the raw materials, and depending on the technological process used, this waste will constitute a mix of various components: bones, skin, viscera and head that can replace valuable resources (Ahmadi et al., 2021).

Traditional fish by-products are: fish meal, fish glue, fish body oils and fish liver oils, sideful, gelatine etc.

Protein concentrate, gelatine, albumin, glue, pearl sideful, peptones, amino acids, protamine, skin are also by-products obtained from fish and fish waste processing (https://agritech.tnau.ac.in/fishery/fish_byproducts).

MATERIALS AND METHODS

This scientific paper presents a review of the most relevant articles in the literature on reducing the amount of fish waste by obtaining by-products, especially value-added products. The effects of the valorisation of fish by-products on related industries have also been studied.

The fish by-products studied come from the processing of capture fish as well as by-catch fish.

RESULTS AND DISCUSSIONS

During fish processing, fish by-products are generated, which are a problem for the food industry and the environment. Converting by-products into different products or using them in other industrial applications can solve the problems and become a sustainable solution.
This literature study presents new starting points of the food innovation process by analysing the actual use of fish waste and fish by-products.
Fish filleting yields can range from 39% to 55%. Differences in fish filleting yields are a reflection of anatomical and structural differences in processed fish, with effects on fish marketability (Okomoda et al., 2021; Nicolae, 2022).
Several value-added products, such as nutraceuticals or enzymes, might be achieved from a wide variety of fish species through various valorisation processes. Fish enzymes, chondroitin sulphate and biopeptides have been shown to be the most suitable by-products to be obtained due to the relatively low impact on the environment and their high selling price. Solid waste treatment technologies for the manufacture of fishmeal, gelatine and chitin should also be considered, which would reduce economic and environmental costs by exploiting unprocessed biomass (Antelo et al., 2015).
By-products of fish (viscera, skin, scales and bones) have been studied as potential raw materials for the separation of value-added compounds with usages in different domains. Hydroxyapatites and collagen could be produced from bones and scales, corresponding to a complete valorisation of these by-products (Ideia et al., 2020).
Zamora-Apodaca et al. (2020), obtained peptide fractions from by-products of various fish species by hydrolysis of soluble collagen. Because they exhibit functional (solubility, emulsification, foaming) and biological (antimicrobial and antioxidant) activity, these fractions represent an alternative for the production of functional foods and products for the pharmaceutical or medical industries.
The components of fish bones are organic and inorganic in nature. In biocompatibility tests by Adamiano et al. (2023), collagen extracted from bones was shown to have potential for cell regeneration. At the same time, different forms of calcium phosphates were obtained whose biocompatibility recommends them for use in cosmetic and biomedical applications.

Food industry

By-products and waste products from fish processing are important sources of protein and various nutrients. When exploiting them, one can consider obtaining myofibrillar fish proteins, homogenized and concentrated (Cercel et al., 2015; 2016).
Antioxidant peptides have been identified in solid waste from fish sauce industrialization. The results showed that fish sauce as a by-product contains a high amount of proteins, i.e. peptides with antioxidant activity.
Most of the peptides in proteins have relatively low molecular weight and strong antioxidant activity (Choksawangkarn et al., 2018).
Fish waste is considered to be a source of high nutritional value due to its rich protein and essential amino acid content. Benhabiles et al. (2012), researched the possibilities of optimizing the process of obtaining fish protein hydrolysate (FPH) from solid waste using enzymatic hydrolysis and crude pepsin.
Hydrolysates of fish proteins can be used as food additives and food ingredients that impart desirable properties to processed foods. These include emulsification, increasing storage stability, foaming or dispersion in creams, sausages, beverages, salad dressings, mayonnaise, etc. Studies on bioactive compounds of hydrolysed proteins are recommended for establishing potential market share, pharmacology and human nutrition, feed additives and/or supplements (Halim et al., 2016).
In a study conducted by Garcia-Moreno et al., in 2014, on five fish species from the Mediterranean Sea, it was found that these species have a lipid content between 4.6 and 25.3%. These can be used as raw material to obtain protein hydrolysates with antioxidant activity.
Three antioxidant peptides were extracted from grass carp skin (*Ctenopharyngodon idella*) by hydrolysis and the use of Alcalase. The peptides obtained, consisting of 5 or 6 amino acids, showed free radical scavenging

activities. Grass carp skin protein hydrolysate has the potential to be used for medicinal purposes and food preservation (Cai et al., 2015).

Ahn et al., in 2014, identified an octapeptide from salmon pectoral fin proteins that exhibited antioxidant activity. The octapeptide has the capacity to remove 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and significantly protect against liver damage produced by hydrogen peroxide in Chang liver cells and DNA damage produced by hydroxyl radicals.

The proteins of fish by-products represents an important bioactive peptides source, and alternatives as ingredients of functional food.

Ennaas et al., in 2015, demonstrated that Gram-negative (*Escherichia coli*) and Gram-positive (*Listeria innocua*) bacterial strains can be totally inhibited by antibacterial peptides from Atlantic mackerel (*Scomber scombrus*) by-products. These results showed that this protein hydrolyzate can be used as an antimicrobial ingredient in both nutraceutical and functional food applications.

The use of oyster mushrooms (*Pleurotus ostreatus*) in making fish burgers from salmon (*Salmo salar*) and striped catfish (*Pangasianodon hypophthalmus*) as a by-product of filleting has proven to be a suitable strategy for technologisation. Thawing losses and thermal processing yield of the products were improved by incorporating 10-15% mushrooms, as the polysaccharides contained in mushrooms improve water retention capacity in frozen products. When mushrooms were added the textural properties (especially hardness) change due to retained water. An improvement in the oxidative stability of frozen burgers was achieved and the acceptability of the products to consumers was not affected. The use of mushrooms resulted in a higher number of microorganisms, but maintained at an acceptable level (Pachekrepapol et al., 2022).

Abdollahi et al., in 2020, evaluated the potential of cross-processing salmon or herring by-products with lingonberry press cake, shrimp peeling by-products and brown seaweed (called "helpers") to minimize lipid oxidation process during alkaline/acid protein isolation with pH change. It was observed that using lingonberry press cake and changing pH significantly reduced aldehydes and hydroperoxides in salmon and herring by-products. The cross-processing concept has the advantage that by using berries and seaweed, lipid oxidation is reduced and the 'clean label' requirements of the food industry are met.

Medicine and pharmaceutical products

Fish by-products is considered an important source of proteins and amino acids with high potential for new nutraceuticals development who can substitute or minimise the possible harmful effects of synthetic drugs. Peptides from protein hydrolysates obtained from fish waste and by-products show bioactivities with antihyperglycemic, anti-inflammatory, antihypertensive, antimicrobial or antioxidant actions. The use of fish waste proteins can minimise the potential pollution of fish waste and develop a high value-added product from a abundant and cheap raw material (Zamora-Sillero et al., 2018).

Cudennec et al. (2008) analyzed the effect of hydrolysates protein extracted from brown shrimp (*Penaeus aztecus*) and blue whiting (*Micromesistius poutassou*) on cholecystokinin released from intestinal endocrine cells. Peptide molecules in fish hydrolysates have been observed to strongly increase cholecystokinin secretion from intestinal endocrine cells. In order to industrially use protein hydrolysates as potential appetite suppressants, should be further studied in both lab rats and humans. The endogenous enzymes obtained from bluefin tuna (*Auxis rochei*) viscera were used to produce bioactive hydrolysates. Ben Maiz et al. (2018) analysed the bioactive and functional characteristics of hydrolysates made at different degrees of hydrolysis. The endogenous enzymatic hydrolysates obtained by low-cost treatment were compared to those made with subtilisin (a commercial enzyme). Functional characteristics of endogenous enzyme hydrolysates were similar or better than subtilisin hydrolysate. Endogenous enzyme hydrolysates have demonstrated antioxidant properties such as free radical scavenging activity and metal reducing activity.

Since hypertension is a high frequency condition and synthetic angiotensin I inhibitors have undesirable side effects, research has been undertaken to obtain angiotensin I inhibitory peptides from fish by-product proteins.

Qian et al., in 2007, isolated angiotensin I, a converting enzyme inhibitory peptide from dark muscle of tuna hydrolysate processed with papain, pepsin, neutrase, alkalase, α-chymotrypsin and trypsin. The pepsin derived hydrolyzate showed the highest angiotensin I inhibitory activity compared to those of the other enzymatic hydrolysates. The peptide had an antihypertensive effect with a maximum decrease in blood pressure detected, 3 hours after oral administration at a dose of 10 mg/kg body weight in hypertensive rats. The results suggest that the peptide derived from dark tuna muscle is a beneficial ingredient for pharmaceuticals or functional foods with an effect against hypertension and its related diseases.

Collagen extracted from Atlantic salmon skin (*Salmo salar*) was hydrolyzed with papain and Alcalase and treated by multi-step isolation. Major fractions of the obtained collagen peptides were collected and their inhibitory activity on angiotensin I converting enzyme was tested. Only two fractions showed inhibitory activity on the conversion enzyme. The results suggested that collagen peptides may be useful as antihypertensive agents and in functional foods (Gu et al., 2011).

Choonpicharn et al. in 2015 demonstrated that gelatin hydrolysate from the skin of Nile tilapia (*Oreochromis niloticus*) can be used as a potential functional antihypertensive agent. Research has shown that trypsin and aromatic hydrolysates have high antihypertensive activity.

Chuesiang and Sanguandeekul, in 2015, evaluated the inhibitory and antioxidant activities of angiotensin I converting enzyme according to hydrolysis time in the production of a protein hydrolysate from tilapia and the effect of varying aminopeptidase concentration. Hydrolysis time and enzyme concentration significantly affected the inhibitory and antioxidant properties of the converting enzyme. 1hour use of 2% (g/g) Flavourzyme® 1000 L for produced the highest levels of free radical scavenging (90.4%), angiotensin I converting enzyme inhibition (83.8%) and metal chelating (91.8%). The functionality of food products can be improved by using protein hydrolyzate as an ingredient.

Ahn et al., in 2012, showed that peptide hydrolysates from by-products salmon of may be useful as an ingredient in functional foods and/or pharmaceuticals. Peptide hydrolysates exhibited antioxidant activity, significantly inhibited the generation of intracellular reactive oxygen species, lipid peroxidation and improved glutathione levels in Chang liver cells and did not show cytotoxic effects on Chang liver cells or macrophage cells. They also exhibited anti-inflammatory activity by inhibiting the formation of nitric oxide and pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-6 and -1β in macrophage cells.

An important source of omega-3 polyunsaturated fatty acids with many benefits for human health is fish oil. It is obtained from by-products of the fishing industry or fish from whole fisheries, from various extraction methods. The oil must be purified to meet acceptable quality standards for human consumption. The refining process removes unwanted compounds: proteins and their degradation products, hydrocarbons, carbohydrates, pigments, mono- and diglycerides, phosphates, steroids, guaranteeing the purity and stability of the oil. During the refining process, phospholipase enzymes are used in the oil degumming process to hydrolyse phospholipids without hydrolysing triglycerides and releases fatty acids into the oil, resulting in less loss. This procedure has been proven to have no negative impact on the environment and has a higher yield compared to chemical degumming. Based on the results obtained, the refining process by enzymatic degumming as the initial stage, is considered a beneficial alternative for improving the physical-chemical characteristics of the oil, while also maintaining the nutritional characteristics (Lamas, 2022).

Ozyurt et al. in 2017 analysed the fatty acid composition and lipid quality of fish oils recovered from sea bass (*Dicentrarchus labrax*) waste, produced with five different strains of bacteria (*Streptococcus* spp., *Lactobacillus brevis, Lactobacillus plantarum*, *Enterococcus gallinarum* and *Pediococcus acidilactici*) and formic acid, in terms of safety for human consumption. In general, it was found that there were no appreciable differences in the unsaturated fatty acid content of the fish oils. Fermented fish waste silage was shown to have a better initial lipid quality than acid fish waste silage lipids. Thus, fish oils recovered from fermented silages can be used as food supplements for humans and animals or food additives.

Fish viscera are a significant source of biomolecules, such as lipids and proteins. Studies have been conducted to evaluate fermentation ensiling as a method of oil recovery from freshwater fish viscera. The total recoverable fat content of the viscera ranged from 19% to 21%; up to 85% of it by fermentation. Regarding oil recovery and fatty acid composition of the recovered oil, fermentation with added lactic cultures (*Pediococcus acidilactici* K7 and *Enterococcus faecium* HAB01) did not differ from natural fermentation. During fermentation the activity of neutral alkaline and acidic protease decreased. Although the degree of protein hydrolysis increased during fermentation, no differences in oil recovery were noted, the highest degree being recorded in the *Pediococcus acidilactici* K7 fermentation (62.3%). The rate of change in degree of hydrolysis decreased as protease activity decreased (Rai et al., 2010).

Ahmadkelayeh et al. in 2022 they studied the extraction, using fish oil depending on the process conditions (temperature, time and oil-to-waste ratio) and pretreatment (drying) of astaxanthin from by-products of Atlantic shrimp (*Pandalus borealis*). A higher yield of astaxanthin in the extract is a consequence of the lower water content in the by-products.

A source of valuable compounds such as hydroxyapatite is the solid waste generated during industrial fish processing. This calcium phosphate is used in the development of biomedical materials due to its biocompatibility with biological and physicochemical properties similar to those of human bone. To improve their bioactive, mechanical and structural properties, hydroxyapatite has been combined with many other chemical compounds. It has outstanding application benefits in the fields of dietary supplements, dentistry, medicine, bone implants and cosmetology. The isolation of hydroxyapatite and the development of products with health benefits are being studied. Hydroxyapatite derived from industrial fish processing, obtained by hydrolysis and calcination, reduces the environmental risks caused by dumping aquatic waste in landfills and adds value to the by-products. (Hernandez-Ruiz et al., 2022).

Animal husbandry

For the production of animal feed (fish meal and fish oil), the aquaculture sector still depends on marine fishing. In 2006-2007, a worldwide survey was conducted on the use of fish oil and fish meal in aquaculture. This survey was started in more than 50 countries and involved 800 feed producers, fishing specialists, researchers, farmers and other people of interest. The survey carried out in 2006 estimated that the use in the aquaculture sector was 835,000 tons of fish oil and 3,724,000 tons of fish meal, respectively 16,600,000 tons of small pelagic forage fish, with a total fish-in fish-out ratio of 0.70. The ratio of small pelagic forage fish per unit of farmed crustacean or fish production showed a steady decrease for all farmed fish species from 1995 to 2006. The biggest declines were in carnivorous fish species such as eel, trout, salmon, salt-water fish and, to a lesser extent, shrimp (Tacon et al., 2008).

Since fishmeal is a major source of protein in aquaculture diets, Saadaoui et al. in 2019 aimed to optimize the hydrolysis of tuna protein to obtain ingredients for these feeds. Free amino acids and fish protein hydrolysates (FPH) have been shown to be useful in increasing feed assimilation and larval development. By optimizing the operating parameters, a fish protein hydrolyzate with the desired molecular weight profile (depending on the specific needs of the farmed species) was made.

The use of tuna by-products as a protein source in tilapia (*Oreochromis niloticus*) aquaculture represents an option to reduce feed costs. Kim et al. in 2019 conducted a study to identify the concentration of heavy metals in tuna by-products. These were found to be safe for use as fishmeal as the safety levels of cadmium and mercury in their composition were not exceeded.

In China, processing fish waste yields up to 650,000 t fishmeal. Even though its protein content is lower (58%) than that of high-quality fishmeal (70%), it can be used to feed low trophic fish. Fishmeal derived from waste of fish is a good source for the culture of various tilapia (*Oreochromis niloticus*) and carp species - common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*). Trout guts from evisceration are a good source of fatty acids for sea bream (*Sparus aurata*). Prior to processing, thermal sterilisation of fish waste is recommended to inactivate microorganisms or pathogens present (Mo et al., 2018).

Ozyurt et al. in 2019 he investigated the impact of the fermentation stage by spraying with strains of lactic and acid bacteria (*Streptococcus* spp*., Enterococcus gallinarum, Lactobacillus plantarum, Pediococcus acidilactici* and *Lactobacillus brevis*) on the formation of biogenic amines in dry and wet fish silos. The silages studied were produced from the processing of whole ponyfish (*Equulites klunzingeri*), crucian carp (*Carassius gibelio*) and by-products of sea bass (*Dicentrarchus labrax*) processing. The results showed that among the biogenic amines, agmatine, spermine, dopamine, spermidine, cadaverine, serotonin and putrescine were predominant in all groups. Wet fish silage and raw fish contained low levels of histamine. No histamine was found in dried fish silage, and a small amount of histamine was found in sea bass by-products. In both wet and dry form, fermented fish silage has potential for use as a protein source and possibly as a probiotic ingredient for animal feed.

An alternative process for the use of fish waste is the use of fish silage, obtained by fermentation with lactic acid, in quail feed. The introduction of this silage in the diet of quails did not negatively modify the production indices, the sensory quality of the meat or the carcass yield. With the inclusion of silage in the diet, the polyunsaturated fatty acid content of quail meat increased. For the reduction of environmental problems and the use of fish waste, the biological production of fish silage is a beneficial biotechnological process (Ramirez et al., 2013).

The valorization of waste of fish in insect rearing is a unique approach that helps aquaculture in achieving the lasting purpose of replacing fishmeal in aquaculture. Chaklader et al. in 2021, showed that Asian perch (*Lates calcarifer*) can better utilize nutrients from diets containing poultry by-product meal mixed with meal from larvae reared on fish waste. Negative effects on the amino acid composition of fish muscles were not recorded, and total protein and albumin were qualitatively increased by these diets. Incorporation of larval meal and poultry meal allowed for the complete substitution of fishmeal in the diet of Asian sea bass, potentially easing the pressure to eliminate marine fisheries and use fish waste, which is much more cost-effective from a circular economy point of view.

Agriculture

Fish waste is a suitable material for composting to ensure sustainable agriculture and its integration into the circular economy.

Radziemska and Mazur in 2015 followed the effects of composting fish waste with manure and mineral fertilizer on the performance and chemical composition of aboveground parts of maize (*Zea mays*). Compost variants composed of fish waste (79.3-80%) to which sawdust, straw, pine bark, lignite were added in varying proportions were made. The highest yield in maize harvesting was obtained from composts containing fish waste, straw and lignite, and fish waste, bark and lignite.

Increased yield and mineral composition of lettuce (*Lactuca sativa*) using compost consisting of 80% fish waste and 20% pine bark was studied by Radziemska et al. in 2018. It was found that adding the fertilizer to the soil increased lettuce leaf production and resulted in significant increases in the nitrogen, magnesium, calcium, sodium, potassium and phosphorus content of the plant leaves.

A potentially valuable use of fish waste is its composting in combination with seaweed and pine bark. This results in a natural and non-limiting fertilizer that can be used successfully in organic farming systems (Lopez-Moschera et al., 2011).

Chemical industry

Artists and craftsmen have used fish glue as a natural adhesive since ancient times. Fish glue can be extracted from various parts of the fish, including the skin, bones, and swim bladder. The Egyptians, as far back as 3500 years ago, briefly described the process of obtaining glue from fish by thermal melting and brush application. Since the 8th century fish glue has been recorded as a painting material for illuminating parchment manuscripts. This product has been used in the preparation of adhesives and binding media, the raw material being commonly available fish protein. Published scientific materials and practical recipe books focus on the use of fish and animal adhesives in the restoration and making of objects, illuminated parchment manuscripts, paintings, icons and other artefacts (Petukhova, 2000).

Fish glue obtained from the swim bladders of tropical fish is used to clarify alcoholic beverages. It is obtained by solubilisation in organic acids and has predominantly collagen in its composition. Because thermal denaturation of collagen in fish glue occurs at 29°C, it is less used than mammalian collagen (Hickman et al., 2000).

The large amount of packaging polluting the environment has prompted the search for innovative solutions. As a result, Florentino et al., in 2022, found that myofibrillar by-product proteins of sawfish (*Pristis pectinata*) and yellow passion fruit peel pectin have suitable chemical characteristics as feedstocks for bioplastic production. The film thus developed can be used as biodegradable primary food packaging.

Scottish researchers have investigated obtaining adipic acid from salmon waste using biological enzymes and then genetically modified bacteria. Adipic acid is a basic ingredient in nylon, but it is also used in other products, including polyurethane-based products used in construction, furniture upholstery, lubricants and pharmaceuticals (https://www.thechemicalengineer.com/news/research-could-see-fish-waste-used-for-nylon-production).

Bioenergy

As the fish farming industry expands rapidly, the by-products resulting from fish oil refining are enjoying increased interest, offering opportunities for bioenergy generation. Some of the by-products of fish oil refining have been studied, such as ethyl monoesters, glycerol and soap. Sarker (2020) investigated the profitability of anaerobic digestion of these by-products as co-substrates with silage of fish (acidified waste of fish). During the experiment, it was found that the volumetric yield of biogas produced (in milliliters) from the co-digestion of monoesters and silage of fish was much higher than that of the monodigester made only with silage of fish. The highest specific biogas yield was obtained of the co-digester with soap stock. The average calculated biogas methane content for all digesters used throughout the experiment was approximately 61%.

Freitas de Medeiros et al. (2019), produced biodiesel using oil resulted from fish processing waste. The experiments conducted used two different methods of biofuel production. Fish residue was thermally treated to extract fatty material, and biodiesel was made by heating it, stirring it, and ultrasonic wave-assisted heating and stirring methods. The best quality biodiesel was produced with heating and stirring, the method having reaction times of 30 and 60 minutes and the yields being 92.37% and 97.95%. The highest biodiesel yield for the ultrasonic method was at a frequency of 20 kHz, with a sonication time of 2 minutes. High concentrations of sodium, potassium and magnesium were found in biodiesel produced from fish waste. In addition, the production of biodiesel from a waste with a high probability of contamination for the environment, such as fish waste, promotes economic and social development.

The possibility of producing biogas from waste of fish as a source of renewable energy was also evaluated. Four concentrations of fish waste (1%, 1.5%, 2% and 2.5%) were used and fermented for 28 days under mesophilic conditions. The economic and energy analyzes estimated an annual production of energy thus produced of 489 MWh. The research results demonstrated that biogas production from waste of fish is a sustainable and viable choice for the proper management of this material and the provision of renewable energy (Cadavid-Rodriguez et al., 2019).

Choi, in 2020, investigated the consequences of anaerobic co-digestion of fish by-product broth mixed with sewage sludge on sludge reduction and biogas production. The 5:5 mixing ratio of broth and sewage sludge produced a recovered energy of 4.1 kWh and generated the highest removal of total solids, volatile solids and chemical oxygen demand. The 5:5 mix ratio is proposed because it can recover the largest amount of energy, can handle larger amounts of fish by-products and is the most efficient. Biogas produced by mixing sewage sludge with fish by-product broth increases the possibility of biogas production using organic waste due to the increased content of methane contained.

Currently, only a fraction of fish waste is used for biofuel production, and most of the waste is dumped on land and in water. Waste fish oil can be used as a potential source for biofuel production after its recovery. Biofuels from fish waste could ease congestion caused by other potential sources of biofuels, due to their low cost. The fatty acid composition of fish varies depending on the type and season, so the quality of the resulting biofuels can vary. The technique used for biofuel production is catalytic esterification, as it can be operated efficiently in a shorter time and at low temperatures. Glycerol is a by-product with extensive requirements in various industries and an extraordinary use in them. In addition, research has demonstrated that biofuels derived from waste of fish have characteristics comparable to biodiesel used according to international standards. Transesterification is a method used to make biofuels and has proven to be a cost-effective method by producing a large amount of biofuel in a short time. However, new strategies need to be developed and implemented to support businesses and fish marketing units by decreasing the amount of discarded waste (Saravanan et al., 2023).

CONCLUSIONS

Fish by-products have great potential for use.

Fish is an important food source for mankind. After processing, a number of by-products are obtained that can become waste or value-added products.

Making more efficient use of by-products of fish is a long-term strategy in the context of increasingly limited resources, high levels of pollution and the desire to increase economic profitability by exploiting unconventional resources.

More research is needed to increase the efficiency of fish by-product recovery and decrease or eliminate existing losses in the fish processing industry.

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THE INFLUENCE OF PRODUCTION TECHNOLOGY PARTICULARITIES ON THE RED WINES CHARACTERISTICS AND QUALITY

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Abstract

In this study, the technological development of two wines obtained from the Feteasca Neagra grape variety was investigated, by the comparison of production methods and the wine's specific characteristics. Although wines were made from the same grape variety, the differences between them are major. Wine production started from the same raw material, but harvested at different maturation periods, reaching different selection and processing methods, and aging methods. As for the production technologies, classical technology, and thermomaceration were used to obtain V-type wine and an artisanal technology for A-type wine (with fermentation in clay amphorae and then continued in oak barrels for 24 months, followed by bottling to aging for a minimum of 12 months). Finally, wine A-type had a lower density of 0.27% and residual sugar of 62.82%, a higher total acidity of 5.77 %, and an alcohol concentration of 13.15%. From the sensory point of view (taste, smell, color, clarity, aroma, general harmony), wine A-type received a higher score.

Key words: *acidity, alcoholic concentration, Feteasca neagra, red dry wine, sensorial characteristics.*

INTRODUCTION

Wine is the drink of all times, appreciated for its qualities and for its positive effect on health when consumed in moderation. Among the advantages of moderate wine consumption is the reduction of the risk of cardiovascular diseases, due to the presence of antioxidants, especially in the case of red wines. Wine is obtained by total or partial fermentation of grapes or juice (Ofoedu et al., 2022; Xiang et al., 2014).

Feteasca Neagra is an old Romanian variety of grapes that has the most spectacular rise in the wine market in Romania, appreciated and requested both by local consumers, but also abroad, and which arouses a special interest on the part of grape producers in growing the areas cultivated with Feteasca Neagra (http://crama-apogeum.ro).

Feteasca Neagră wine has a discreet but very specific olfactory character, suggesting the smell of dried plums, and when the wine is older, shades of cinnamon appear. In perfecting the sensory properties, the substances involved in the formation of the taste, smell, color, and other characteristics (the degree of foaming and game of the wine, the clarity), being an exclusive effect of fermentation or a mechanical operation (https://tohaniromania.com).

The quality and the name (brand) of wine are its main assets, and influence consumer behavior and choice (Veríssimo et al., 2021; Oliveira et al., 2019a; Oliveira et al., 2019b).

The quality of a wine can be determined by many factors, including its chemical composition (concentration of sugars, acidity, pH, alcohol level, polyphenol content), as well as the technology of obtaining and aging process (Hopfer et al., 2015).

The chemical composition of wine is one of the main indicators of its quality and influences its acceptability and appreciation by consumers; studies have shown that high levels of polyphenols, especially tannins, are associated with higher wine quality (Cory et al., 2018).

Wine production technology can also influence its quality. The use of well-ripened grapes doubled by traditional methods of fermentation, and aging in oak barrels can improve its quality and sensorial characteristics (Ribéreau-Gayon, 2021).

Aging is an important production phase for high-quality wines, because of the synthesis of certain substances involved in aroma, taste, smell, general appearance, and particular mark (label) of wine. Aging wine in wooden barrels or bottles can improve the aroma and taste of the wine through complex chemical reactions that take place during this process (Boulton et al., 1996).

The evaluation of wine quality is based on organoleptic analysis, which involves the evaluation of the taste, smell, and appearance of the wine by the human senses. This evaluation method can be subjective and can vary depending on the tasters' individual tastes, but using highly trained specialists in wine tasting and evaluation can offer a good perspective on the value of a wine type (Barbe et al., 2021).

Therefore, in order to obtain a superior quality wine, it is important to pay attention to both its chemical composition and the production and aging process.

The chemical composition of dry red wine can influence wine quality through several factors. The level of sugars can influence the taste and aroma of the wine; a low concentration of sugars can result in a dry wine, while a higher concentration can result in a sweet wine. The alcohol level can also influence the taste and aroma of the wine, with too high an alcohol level leading to a burning sensation in the mouth and an unpleasant aftertaste (Miao, 2022).

Another important factor is acidity, which can influence the taste and structure of the wine; a wine with high acidity can be perceived as sour, while a low-acidity wine can be perceived as lacking brightness and character (Scutarașu et al., 2021).

The content of polyphenols, especially tannins, can also play an important role in the quality of dry red wine. These are organic compounds that can contribute to wine aromas and tastes, as well as texture and structure. Studies have shown that high levels of polyphenols, especially tannins, are associated with higher wine quality (Hosu et al., 2016; Nemzer et al., 2022).

The chemical composition of wine can also influence organoleptic properties such as aroma and taste. For example, the concentration of volatile compounds in the wine can influence its aroma, and the level of sugars can influence the sweetness and body of the wine (Escudero et al., 2007).

The taste is a basic indication in the sensory assessment, the elements that are evaluated in the case of this characteristic are the intensity and quality of the taste, the harmony of the taste components and the softness of the taste, the alcoholic strength, the acidity, the sweetness, the astringency.

The substances involved in taste formation are ethyl alcohol (gives the wine viscosity, reduces the acidic taste, increases the degree of sweetness, and the taster's olfactory sensitivity), glycerol (reduces the sour-pungent taste, making the wines “softer”, also contributing the to the preservation of odorous substances); sugars (hexoses, pentoses); organic acids (gives wines their sour, harmonious taste, i.e. "vibrancy"), the tannin content. A study published in the journal Red Wine Technology analyzed aroma compounds in red wines and found that the level of tannins and phenolic compounds were the most important factor influencing the wine's sensory characteristics, including taste. Alcohol level and acidity were also associated with wine sensory characteristics such as fullness and freshness of taste (Escudero, 2007; Rauhut, 2019).

The substances involved in the formation of the odor are found in small quantities, from µg/l to a few mg/l. The most odoriferous compounds in wine are those that are not only found in large quantities but also have a higher vapor pressure as well as a higher odor intensity (terpene compounds: geraniol, neralol, α-terpinol, linalool, limonene, citronellol). The level of terpene compounds is 0.3-3.5 mg/l in aromatic varieties; 0.5 mg/l for mildly aromatic varieties; 0.2 mg/l for unflavored varieties (Meilgaard et al., 2015).

The smell of dry red wine can be described by various aromatic notes, such as black fruits (strawberries, raspberries, black currants), berries, spices (black pepper, vanilla, cinnamon), chocolate, coffee, or wood (depending on the type of barrels in which the wine was matured). These aromas are influenced by the chemical composition of the wine, but also by the fermentation and aging processes. Volatile aromatic compounds such as esters and aldehydes are produced during fermentation and can contribute to the wine's aromatic notes. In addition, the acidity level and pH of the wine can affect the aromatic notes, with a higher pH reducing the intensity of certain aromas (Ieri, 2021; Ribreau-Gayon, 2021).

MATERIALS AND METHODS

The study was carried out in a well-known winery in Romania and the influence of the use of different technologies for processing grapes from the Romanian variety Feteasca Neagra on the final quality of the obtained dry red wine was followed.

The research was carried out over two years (2020-2021), tracking three batches of V-type wine, obtained by classical technology and thermo-maceration, and one batch of A-type wine, obtained by artisanal technology.

Figure 1. Clay amphorae used for fermentation of grape juice to obtain A-type wine

Starting from the goal to enlighten the humble Feteasca Neagra grape variety, the winery brought back to life a 2000 years old unique Romanian fermentation process. The grapes come from an old vineyard, specially arranged and cared for, located in one of the best wine-growing areas in Romania. The plantation is 50 years old, on calcareous, has poor soil and benefits from small amounts of precipitation.

The production yield is small, 5-6 tons/ha, harvested by hand. The grapes are selected from the vineyard, picked in the optimal period, and transported with great care to the winemaking place, where fermentation takes place in clay amphorae (Figure 1), followed by a period of 24 months, during which the young Feteasca Neagra rests in the barrel; then, the wine is aged for at least 12 months in glass bottles, for its elegant maturity notes, expressed by an explosion of aromas (oak, vanilla, chocolate, coffee), soft, velvety tannins, elements which denote the value of a special, rare wine.

The physicochemical parameters analyzed during the fermentation process were: density, residual sugars, alcohol content, free and total SO_2 content, total and volatile acidity.

The two wines were also analyzed from the sensorial point of view.

The results were processed statistically by classical methods.

RESULTS AND DISCUSSIONS

The physicochemical parameters analyzed during the fermentation period for the two wines are shown in Tables 1 and 2, respectively in Figure 2.

The initial density was 1.107-1.113 g/cm^3 for V-type wine and 1.117 g/cm^3 for A-type wine.

During fermentation, the density decreases, so at the end of the fermentation period, V-type wine has a density between 1.009-1.013 g/cm^3, and A-type wine, 0.994 g/cm^3.

References from the literature indicate values of the density of dry red wine at the end of fermentation of 0.992-0.998 g/cm^3 (Cabernet Sauvignon: 0.992-0.996 g/cm^3, Merlot: 0.990-0.996 g/cm^3, Pinot Noir: 0.993-0.996 g/cm^3). This can vary depending on many factors, such as grape variety, fermentation process, and other processing conditions (Boulton et al., 1999).

Jackson (2020) shows that the density is 0.990-1.020 g/cm^3, which can also vary depending on the temperature at which it is measured and the atmospheric pressure.

The content of sugars in V-type wine decreases by 93.2-95.2 % compared to the initial values; for A-type wines, the decrease is 88.89-90.25 %

The literature states that the amount of residual sugar in dry red wine at the end of the fermentation process can vary depending on the type of wine, fermentation conditions, and other factors. In general, the residual sugars in dry red wine at the end of fermentation can be 2 g/l or less, indicating that most of the sugars have been converted to alcohol. However, some dry red wines can have higher residual sugar, up to 5-6 g/l or even more, to achieve a certain sweetness or balance in taste (Blazquez Rojas, 2012)

Table 1. The evolution of must parameters followed by days of fermentation in 2021, V-type wine

Fermentation day	Batch 1			Batch 2			Batch 3		
	Density (g/cm^3)	Temperature (°C)	Residual sugars (g/l)	Density (g/cm^3)	Temperature (°C)	Residual sugars (g/l)	Density (g/cm^3)	Temperature (°C)	Residual sugars (g/l)
1	1.113	19	270	1.111	20	264	1.107	16	252
2	1.113	20	270	1.109	20	260	1.082	15	190
3	1.110	18	262	1.094	20	223	1.075	16	172
4	1.097	17	228	1.071	23	167	1.065	20	150
5	1.076	20	177	1.048	20	106	1.056	18	126
6	1.068	20	156	1.025	23	72	1.046	20	100
7	1.038	23	83	1.015	24	45	1.032	20	78
8	1.030	22	75	1.009	20	34	1.013	23	44
9	1.019	23	50	1.005	20	22	1.008	18	32
10	1.011	20	39	1.001	19	16	1.004	19	20
11	1.006	20	28	0.997	20	8	1.002	20	16
12	1.003	19	18	0.994	20	3,20	0.999	20	12

Table 2. The evolution of must parameters followed by days of fermentation, A-type wine

Fermentation day	2020			2021		
	Density (g/cm^3)	Temperature (°C)	Residual sugars (g/l)	Density (g/cm^3)	Temperature (°C)	Residual sugars (g/l)
1	1.117	16	277	1.117	20	279
2	1.103	19	245	1.112	19	267
3	1.060	18	136	1.099	18	235
4	1.048	19	106	1.086	19	203
5	1.037	20	94	1.075	19	174
6	1.030	17	80	1.069	18	159
7	1.022	17	52	1.057	19	129
8	1.010	17	35	1.050	18	111
9	1.007	20	30	1.043	19	93
10	1.000	19	14	1.021	18	52
11	0.998	20	10	1.011	19	38
12	0.997	20	8	1.006	20	28
13	0.996	20	6	0.999	19	13
14	0.995	20	5.1	0.994	18	3.1
15	0.994	20	2.7			

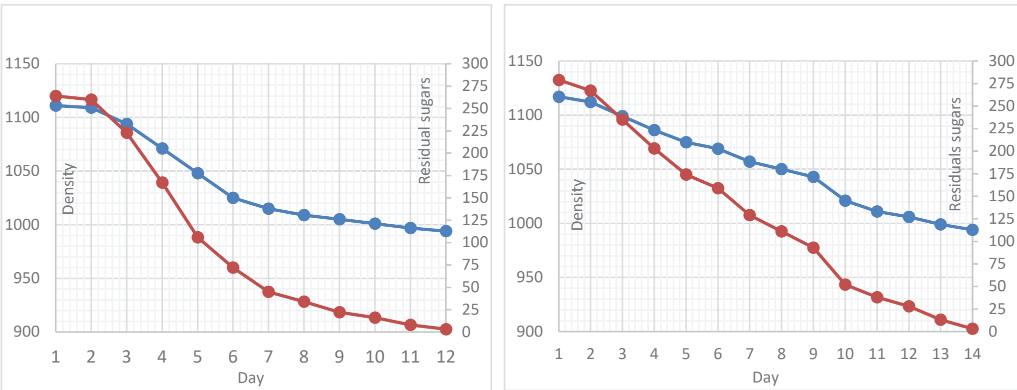

Figure 2. Evolution of density and residual sugars: V-type wine, batch 2/2021; A-type wine, 2021

V-type wine, obtained by classical technology, respectively by thermomaceration, was analyzed sensory (Table 3) and from the point of view of physicochemical parameters (Table 4).

Table 3. Sensory characteristics of wine V

Feteasca Neagra V-type	Sensory characteristics			
	The clarity	Color	Flavor	The taste
Classical	Clear, without sediment and foreign inclusions	Intense red	Characteristic of the variety with nuances of black currant and dried fruit	The characteristic taste of the blackberry variety is generally balanced, consistent and vigorous. The right acidity, relatively persistent
Thermomacerated	Clear, without sediment and foreign inclusions	Red, very intense	Characteristic of the variety with nuances of black currants and dried fruits, especially prunes	The taste characteristic of the variety. The right acidity, relatively persistent and "baked" taste

The analysis of the sensory properties of V-type wine obtained by the two processes shows that they correspond to the characteristics of young wines. Both processes were conducted on wines with good clarity, without sediment and mechanical inclusions. The color is different in intensity, being weaker in the case of the classic process than in the thermomaceration one. The aroma of wines obtained from Feteasca Neagra is pleasant, complex, and original. The taste is generally consistent and vigorous, with a suitable acidity, characteristic of the variety, which intervenes beneficially, leaving behind a pleasant and persistent memory.

These values of the physicochemical parameters (Table 4) characterize the young wine and express their future behavior, respectively the potential that will be reached. It is noted that the wines have a high alcohol content, a welcome factor that can ensure physicochemical stability. The titratable acidity is within the permissible limits for red wines, and the volatile acidity shows relatively high values for young wines, but which do not exceed the norms. The mass concentration of sulfur dioxide is relatively low.

Table 4. Physicochemical characteristics of V-type wine

Indices	V-type wine (classical)	V-type wine (thermomaceration)
Alcohol concentration (% vol.)		14
Mass concentration of sugars (g/dm^3)	2.6	2.8
Mass concentration of titratable acids (g/dm^3)	7.7	7.1
Mass concentration of volatile acids (g/dm^3)	0.90	0.93
Mass concentration of free sulfur dioxide (mg/dm^3)	26	13
Mass concentration of total sulfur dioxide (mg/dm^3)	80	65.5
Mass concentration of iron (mg/dm^3)	6	6
Ph	3.72	3.76
Total polyphenolic index, TPI (units)	62.6	68.3
Color intensity, I_c (unit.)	16.73	21.04
Color purity (dA)	53.5	47.4
Anthocyanins (mg/dm^3)	163.8	193.8

The vinification of Feteasca Neagra grapes through the two technological processes led to different values, both of the chromatic and phenolic characteristics. The wine obtained by thermomaceration showed a higher level of color intensity (+9.1%) and a higher polyphenolic index (+25%).

The average final density of the two analyzed wines was significantly equal (only 0.2% higher in V-type wine), and the remaining sugars were 2.68 times higher in V-type wine.

Total sulfur dioxide had an average of 54.1 mg/l in A-type wine and with 18.5% lower in V-type wine.

The literature states that the level of total sulfur dioxide (total SO_2) concentration in dry red wine can vary depending on many factors, such as the grape variety, the location of the crop, the winemaking technique, and the level of preservation. In general, the concentration of total sulfur dioxide in dry red wine can be from 10 to 40 mg/l for regular wines, and up to 160 mg/l for premium wines.

It is important to note that total SO_2 levels in wine are regulated by law and the maximum allowed for dry red wine in the European Union is 160 mg/l. In the USA, the maximum level allowed for dry red wine is 350 mg/l (https://www.oiv.int/; https://www.fao.org/).

Table 5. The final values of the wort parameters (when it is drawn)

Specification			Density (ρ)	Residual sugars (g/l)	Alcohol (%)	Free SO_2 (mg/l)	Total SO_2 (mg/l)	Total acidity (g/l)	Volatile acidity (g/l)
Wine V	2020	Charge 1	995	5.20	14.1	12.6	40.8	5.74	0.29
		Charge 2	998	10	12.5	19.4	36	5.50	0.30
		Charge 3	994	2.4	15.7	16.8	33.6	4.72	0.34
	2021	Charge 1	994	3.2	15.3	12.4	48	4.64	0.34
		Charge 2	1000	14	15	17.8	56	4.59	0.37
		Charge 3	999	12	14.1	15	50.21	4.98	0.40
Wine A	2020	Charge 1	994	2.7	16.2	19.4	47.80	5.39	0.31
	2021	Charge 1	994	3.1	16.5	24.30	60.4	5.24	0.37

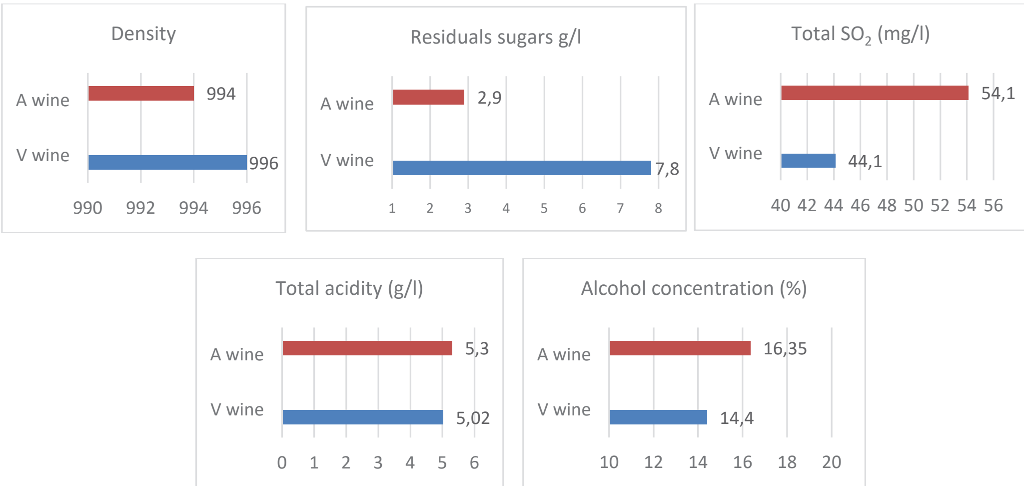

Figure 2. Average final physicochemical parameters of the studied wines

It should be noted that there are differences between free sulfur dioxide (free SO_2), which can be toxic in high concentrations, and total sulfur dioxide (total SO_2), which includes both free SO_2 and bounded SO_2, which is not toxic. Most of the SO_2 content in dry red wine is in bound form and therefore poses no health risk to consumers.

The final total acidity was 5.3 g/l in A-type wine and slightly lower in V-type wine (5.02). The values found in scientific studies are between 4.5-7.5 g/l (Ofoedu et al., 2022; Veríssimo et al., 2022, Hopfer et al., 2015).

The final alcoholic concentration recorded average values of 16.35% for wine A and 14.4% for wine V.

Literature cites an alcohol content of dry red wines between 11.5% and 13.5% vol in Italy, between 12 % and 14 % vol in France, and between 13.5% and 15.5% vol (Oliveira, 2019a şi 2019b; Xiang, 2014).

Studies have shown that the alcohol level can influence the taste and aroma of dry red wine. In general, higher alcohol content can give the wine a warmer feel and a more pronounced sweetness, but it can reduce the complexity and subtlety of the aromas and tastes. Conversely, lower alcohol content can make the wine more subtle and delicate in taste and aroma, but can also lead to a cooler mouthfeel (https://www.oiv.int/).

For a complete analysis, we continued with the evaluation of the sensory properties, since the wine cannot be qualitatively judged only according to the chemical composition or the microbiological state, but also according to the sensory quality.

During the tasting process, the samples were taken out of the barrel into clean bottles with labels, 1-2 hours before the actual tasting, avoiding aeration of the wine. The tasting temperature was around 15-17°C. The longer maceration period can lead to a deeper and more intense color of the wine.

Related to the color of the young dry red wine, the anthocyanin pigments in the grapes are the main determining factor. The pigments are present in the skin of the grapes and are released during the fermentation process. Younger red wines are usually more intense in color and have purple or ruby tones. As the wine ages, the color may change to darker shades such as brown or orange. This is due to the oxidation of anthocyanins and the formation of more stable pigments (Barbe et al., 2021; Oliveira et al., 2019a).

The color of dry red wine can influence taste perception. One study showed that participants described red wine with deeper shades of red as sweeter and more aromatic, while wines with lighter shades were perceived as drier and sourer. (Oliveira et al., 2019a).

Regarding the taste of dry red wine, the specialized literature describes it as being characterized by intense aromas of red and black fruits, such as cherries, currants, raspberries, plums, or blackberries, together with notes of spices, vanilla, or chocolate. The taste can be described as dry or slightly sweet, with moderate acidity and well-balanced alcohol strength (Meilgaard, 2015).

Following the sensory analysis, it was found that V-type wine has an intense ruby color. The wine is extremely generous, with aromas of jam and ripe cherry, plus notes of black truffle, cloves, and dry tobacco leaves.

On the palate, there is a remarkable voluptuousness, which meets an aroma of red berries, black cherries and sour cherries. The supple tannins integrate perfectly into the complexity of this wine. The total average score obtained by this wine was 90 points.

In the case of A-type wine, the color was ruby red, dense, and bright. Primary aromas of plum, blueberry, and blackcurrant are complemented by hints of licorice and coffee. The spices that define the wine's character, pepper, cloves, and vanilla, are integrated and complete the aromatic picture.

The taste is soft, with soft and juicy tannins. The average score obtained by this wine was 94 points.

CONCLUSIONS

The results of the experimental study which analyzed the evolution of the same raw material processed by two different technological processes highlighted the impact on the finished product and notable differences in terms of the physicochemical parameters and the seasonal properties of the two wines.

These differences are directly influenced by the technological process.

V-type wine, processed in a classic, industrial system (the must be obtained by pressing, and the fermentation is done in stainless steel vessels, without a subsequent aging period) finally had a lower SO_2 content, a higher residual sugar, and a smaller amount of alcohol. As the level of carbohydrates decreases, the level of alcohol concentration increases.

A-type wine was obtained in an artisanal way, benefiting from a cold maceration of the grapes, a longer fermentation period in clay amphorae, which led to a lower residual sugar, a higher amount of alcohol and SO_2, but also a slightly higher acidity. They ensure the wine an increased shelf life, without the need for interventions on the values.

Following the performance of the sensory analysis test, the fullness of the wines, the perceived intensity, and strength, the outline of the complexity, and also the specific aromatic notes from which the uniqueness of wine A was deduced.

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QUALITY CHARACTERISTICS OF YOGURT FROM BUFFALO MILK SUPPLEMENTED WITH ARONIA (*Aronia melanocarpa*) JUICE

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Abstract

Yogurt was prepared from buffalo milk supplemented with 3% and 5% Aronia (Aronia melanocarpa) juice. The mineral and fatty acid composition, free amino acid composition, vit. B1, B2, B6 and antioxidant activity were investigated. Buffalo yogurt produced with 3% aronia coagulated in a shorter time (135 min) compared to natural (control) yogurt and the one produced with 5% aronia (158 min). Buffalo yogurt produced with 5% aronia juice has the highest content of potassium (1004 mg/kg) and zinc (5.28 mg/kg) and the lowest of calcium, magnesium and manganese compared to the control yogurt and yogurt with 3% aronia addition. Aronia supplementation increased the amount of unsaturated fatty acids in buffalo yogurt by 5.7% (3% aronia) and 7.3% (5% aronia), respectively. Polyunsaturated fatty acids increased by 15.7% in 3% aronia yogurt and 22.6% in 5% aronia yogurt respectively, compared to natural buffalo yogurt. Yogurt produced with 5% aronia juice has the highest antioxidant activity and also has a higher content of vitamins B1, B2 and B6 compared to the control yogurt and yogurt with 3% aronia.

Key words*: antioxidant activity, aronia, buffalo milk, fatty acid composition, yogurt.*

INTRODUCTION

Yogurt is a wide-spread fermented dairy product and has long been recognized as a functional food with desirable health effects (Rul, 2017). It is rich of nutrients with high biological value as well as essential micronutrients and beneficial microorganisms. Although dairy products have their obvious health benefits, however, fermented dairy products, including yogurt, are not documented as a rich source of bioactive compounds like antioxidants and polyphenols (Sheikh et al., 2022)

On the other hand, people are becoming more aware of their nutrition and demand of novel functional foods shows a steady rise in the last few decades. Yogurt is considered to provide a perfect matrix where different substances can be introduced and in recent years numerous experiments has been conducted in attempts to increase its biological activities. The most common are the supplementations of yogurt with different fruits, herbs and fibers because of their natural origin and potential advantages for human health.

Fruits of aronia (*Aronia melanocarpa*) are known to be a great source of polyphenols, including anthocyanins, flavonols, flavanols, proanthocyanidins, and phenolic acids (Oszmiański & Lachowicz, 2016; Tolić et al., 2017) and has also been used to enhance the benefits of yogurt. Several studies have reported that fortification of cow and goat yogurt with fruits of aronia (black chokeberry) significantly increased its total phenolic content, antioxidant activity (Dimitrellou et al., 2020) and percentage of polyunsaturated fatty acids in it (Boycheva et al., 2011). The abundance of bioactive components in aronia is linked with preventative and healing capacity against many chronic diseases. Its fruits display antidiabetic (Qin & Anderson, 2012; Lipińska & Jóźwik, 2017), anti-inflammatory (Zapolska-Downar et al., 2012) and anticancer activity (Cvetanović et al., 2018). Aronia is also known to reduce oxidative stress by cleaning free radicals (Dietrich-Muszalska et al., 2014) and can exhibit anti-toxic effects against numerous harmful substances (Borowska & Brzóska, 2016).

Although the yogurt production worldwide is predominated by cow milk, over the last years there is an increased interest in using buffalo milk as a raw material. Compared with bovine milk, buffalo milk is characterized by higher levels of calcium, fat, lactose, protein, casein, and ash content, and is a good source of minerals, such as magnesium, potassium, and phosphorus (Abd El-Salam & El-Shibiny, 2011;

Abdel-Hamid et al., 2017; Khan et al., 2017). It also contains more tocopherols, vitamin A (Basilicata et al., 2018) and is associated with lower risk of allergies than cow`s milk (Sheehan & Phipatanakul, 2009). As a result, buffalo milk has been used with considerable success for producing of numerous dairy products, including yoghurt (Abdel-Hamid et al., 2017). The combination of buffalo yogurt high nutritional value with bioactive phenolic compounds in aronia fruits is an option for creating a natural functional food with beneficial effect on human health.

The purpose of this study is to investigate the effects of aronia juice on antioxidant activity, fatty acid composition, content of free amino acids, minerals, vitamins B1, B2, B6 of buffalo yogurt.

MATERIALS AND METHODS

Fresh milk and starter cultures. Fresh raw buffalo milk and a starter culture, containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Lactina 17, Bankya, Sofia, Bulgaria) ready for direct vat inoculation were used for yogurt preparation. The buffaloes' yogurt was prepared in laboratory conditions.

Yogurt preparation. The milk was pasteurized (95°C/30 min), cooled to 45°C, and inoculated with 1.5% yogurt culture consisting of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Lactina 17, Bankya, Sofia, Bulgaria). The raw milk was divided into three lots - control and 2 experimental. Prior to adding the starter, 3 and 5 g/kg fruit juice from aronia was added to the experimental milk samples. The samples were then cultivated at 42°C until coagulation, then cooled and stored in a refrigerator at 4-6°C.

Fatty acid composition

The extraction of milk fat was done by the method of Rose-Gottlieb using diethyl ether and petroleum ether (Methodenbuch, Bd. VI VDLUFA-Verlag, Darmstadt, 1985). After that the solvents were evaporated on a vacuum-rotary evaporator. Sodium methylate (CH_3ONa) was used for obtaining methyl esters of the fatty acids. The fatty acid composition of raw milk and yoghurt was determined by gas chromatography "Clarus 500" with flame ionization detector and column Thermo Scientific, 60 m, ID 0.25 mm, Film: 0,25 µm.

Vitamins determination

The determination of vitamins B1, B2 and B6 was carried out using VitaFast® by extracting the respective vitamin from a homogenized sample and diluting the extract. The diluted extract and standards were applied to the wells of a microtiter plate coated with *Lactobacillus fermentum* (B1), *Lactobacillus rhamnosus* (B2) and *Saccharomyces cerevisiae* (B6), respectively.

Free amino acid analysis and mineral composition

The free amino acid was done by EZ: fast Amino Acid Analyis Kit from Phenomenex Inc for GC/FID.

The macro- and microelement composition were determined after dry incineration of the sample, its mineralization in a muffle furnace at 550°C and preparation of a hydrochloric acid solution. Mineral composition was determined by atomic absorption spectrophotometer "Perkin-Elmer 380".

Determination of antioxidant activity

Determination of Total Flavonoid Content (TFC)

TFC was determined via the aluminium trichloride method, using catechin as reference material (Dinev et al., 2021). In brief, 1.0 mL sample (of skimmed milk with protein removed or juice diluted by deionized water 1:4) with, 0.3 mL 5% $NaNO_3$, and after 5 min, 0.3 mL 10% $AlCl_3$ were added in a 10 mL volumetric flask containing 4.0 mL deionized water in this order. After 6 min, 2.0 mL of 1 M NaOH solution was added and the total volume was adjusted up to 10 mL using deionized water. The suspension obtained was homogenized and centrifuged (4000 x g) at room temperature. The absorbance of the supernatant was measured against a prepared reagent blank at λ = 510 nm on a Thermo Scientific Evolution 300 spectrophotometer. Standard solutions of (+)-catechin hydrate (Sigma Aldrich, St. Louis, MO, USA) in the concentration range from 10 to 100 mg/L were used to plot the calibration curve. The total flavonoid content was expressed as µg catechin equivalent (CE) in 1 L (µg CE/L). Each sample was analysed in triplicate.

Determination of Radical Scavenging Activity by DPPH Method
DPPH (1,10-diphenyl-2-picrylhydrazil-radical) was purchased from Sigma-Aldrich (St. Louis, MO, USA). This substance has a single electron on the nitrogen atom and its solution in methanol has an absorption maximum at λ = 517 nm. The mechanism of the DPPH method is based on the reaction between the test compound and DPPH-radical, wherein the potential free radical scavengers reduce DPPH-radical (violet solution) to a yellow coloured 1,10-diphenyl-2-(2,4,6-trinitrorhenyl) hydrazine by donating a hydrogen atom. The method described by Dinev et al. (2021) was applied to measure the radical scavenging potential of the samples of skimmed milk with protein removed, and juice diluted by deionized water 1:10 In brief, 0.1 mL of each sample was added to 3.9 mL of 100 mM solution of DPPH in methanol. The absorption of the supernatant obtained by centrifugation (4000 x g) at room temperature at λ = 517 nm was measured 30 min later on a Thermo Scientific Evolution 300 spectrophotometer. The results were calculated using regression analysis from the linear dependence between the concentration of Trolox and the absorption at 517 nm. The results were expressed as µmol Trolox equivalent (TE) in 1 L (µmol TE/L). Each sample was analysed in triplicate.
Statistical analysis. For statistical analysis, statistical software IBM SPSS-Inc., 2019, (SPSS Reference Guide 26 SPSS, Chicago, USA) was used.

RESULTS AND DISCUSSIONS

Free amino acid
In the process of fermentation of yogurt proteins are partially hydrolysed into peptides and free amino acids due to the proteolytic activities of lactic acid bacteria (Germani et al., 2014). The total amount of amino acids in experimental samples raised significantly compared to the control, being 2.20 times higher in 5% aronia samples and more than 3 times higher in these with 3% aronia (Table 1). The same trend was observed with the quantity of essential amino acids - 4.78 and 3 times higher in 3 and 5% supplemented yogurt respectively. In our opinion the added aronia juice provided lactic acid bacteria (LAB) with beneficial substances and enhanced their growth, which resulted in bigger quantities of free amino acids in enriched yogurt. The studies of Nguyen & Hwang (2016) and of Boycheva et al. (2011) showed that yogurt containing aronia juice showed higher LAB counts than plain one. Another possibility for higher levels of amino acids in fruit samples is the ability of polyphenol compounds to interact with milk proteins (O'Connell & Fox, 2001). The main representative of essential amino acids in three samples was lysine with its amount increasing from 30.32 mg% in control to 74.39 mg% and 140.01 mg% in 5% and 3% aronia enriched yogurt.

Table 1. Free amino acid content in plain and aronia supplemented buffalo yogurt, mg %

Free amino acids	Control	Aronia 3%	Aronia 5%
	Mean±SEM	Mean±SEM	Mean±SEM
Alanine	12.53^{a*}±0.10	26.70^{a}±0.07	21.53^{a}±0.27
Glycine	2.57^{a}±0.18	6.63±0.32	6.91^{a}±0.12
Valine	6.98^{a}±0.04	19.19^{a}±0.06	17.02^{a}±0.03
Leucine	21.65^{a}±0.18	57.91^{a}±0.06	45.32^{a}±0.67
Isoleucine	6.34^{ab}±0.03	17.36^{a}±0.28	14.84^{b}±0.07
Threonine	2.34^{a}±0.06	59.45^{a}±0.27	48.07^{a}±0.37
Serine	41.46^{a}±0.26	38.80^{a}±0.06	30.61^{a}±0.17
Proline	11.84^{ab}±0.09	18.60^{a}±0.16	18.91^{b}±0.03
Asparagic acid	8.07^{a}±0.03	20.47^{a}±0.41	21.88^{a}±0.05
Asparagine	9.68^{a}±0.11	18.51^{a}±0.20	11.86^{a}±0.10
Methionine	0.28^{a}±0.01	0.89^{a}±0.00	0.25^{a}±0.07
Glutamic acid	4.22^{a}±0.05	6.65^{a}±0.07	3.89^{a}±0.06
Phenylalanine	5.65^{a}±0.03	24.70^{a}±0.05	16.95^{a}±0.04
Glutamine	2.62^{a}±0.05	12.70^{a}±0.07	7.82^{a}±0.18
Histidine	3.76^{ab}±0.11	9.09^{a}±0.06	9.57^{b}±0.12
Lysine	30.32^{a}±0.04	140.01^{a}±0.36	74.39^{a}±0.13
Tyrosine	5.99^{a}±0.08	46.14^{a}±0.58	20.11^{a}±0.01
Tryptophan	9.47^{a}±0.02	74.99^{a}±0.09	31.82^{a}±0.09
Cysteine	-	0.77^{ab}±0.01	0.29^{a}±0.01
Arginine	7.64^{a}±0.05	28.62^{a}±0.25	21.34^{a}±0.25
Total	193.48	628.18	425.79

^{*a,b} - $p<0.05$

The branched chain amino acids (BCAAs)- valine, leucine and isoleucine belong to the essential ones and are reported to play a key role in metabolism of glucose, lipids, protein synthesis and intestinal health (Nie et al., 2018). Their levels in the organism can also serve as early signals for development of some chronic diseases (Newgard et al., 2009). The biggest amounts of BCAAs, with main representative leucin was recorded in 3% aronia yogurt as every particular amino acid increased its quantity more than twice compared with the control sample content.

Among the sulfur amino acids (SAAs) methionine and cysteine are considered to be principal ones as the former is classified as essential and the latter as semi-essential one. Recently, numerous studies have investigated SAAs` significance for protein metabolism and synthesis and their role as a precursor of important molecules (Métayer et al., 2008). The content of methionine decreased slightly in 5% aronia yogurt compared with the control, while there was considerable raise in 3% yogurt - from 0.280 mg% to 0.890 mg%. Cystein wasn`t detected in the plain yogurt, however in the supplemented ones it`a amount varied from 0.290 mg% in 5% to 0.770 mg% in 3% aronia yogurt.

The greatest increase of quantity compared to the plain yogurt was observed with threonine – more than 20 times in 5% supplemented and 25 times in 3% one. As an essential amino acid threonine is important for lipid metabolism in liver and for protein synthesis in human body (Tang et al., 2021).

As to non-essential amino acids the main representative in control sample and in 5% supplemented yogurt was serine - 41.46 mg % and 30.61 mg % respectively, while in 3% yogurt it was tyrosine - 46.14 mg %. The quantity of both essential and non-essential amino acids increased in aronia supplemented yogurt compared to the plain one, more significantly in 3% enriched samples (Figure 1).

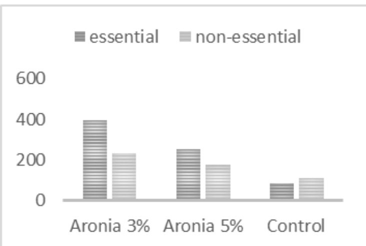

Figure 1. Essential and non-essential amino acids in yoghurt, mg%

Fatty acid profile of yogurt

The results showed that the amount of saturated fatty acids (SFA) in experimental samples was slightly lower than in control (Table 2). Similar data were reported by Boycheva et al. (2011) about goat yogurt, supplemented with 5%aronia juice. As expected, predominant in SFA was palmitic acid with a maximum concentration in 5% yogurt - 29.54%, while smallest percentage was reported by tridecyl acid - 0.21% in both enriched yogurts.

Highest concentration of unsaturated fatty acids (UFA) was observed in 5% aronia yogurt. The quantity of monounsaturated fatty acids (MUFA) with main representative oleic acid raised insignificantly compared to the plain yogurt. Largest amount of polyunsaturated fatty acids (PUFA), which are considered to have prophylactic effect against some chronic diseases (de Caterina & Zampolli, 2001) was also obtained in 5% aronia yogurt with a significant increase in the percentage of linolenic acid, that was 2.2 times higher than in control. The linolenic acid is known to have cardiovascular - protective, neuroprotective, anti – inflammatory and anti - oxidative effects (Kim et al., 2014) The recent study of Tasinov et al. (2022) showed that main fatty acids in aronia juices on Bulgaria market were the nonessential stearic and essential linoleic and linolenic acids. Therefore, we presumed that the highest amount of linolenic acid in 5% aronia samples was due to the added juice. However, our results differed from these of Boycheva et al. (2011) where quantities of linolenic acid in plain goat yogurt and in 5% aronia supplemented one were quite lower and almost the same - 0.63% and 0.65% respectively. On the other hand, the concentration of conjugated linoleic acid (CLA) decreased in fortified samples, more significantly in 5% one. Compared to the results of Kowaleski et al. (2020) about 10% strawberry enriched cow yogurt, containing 1.29% CLA, the aronia supplemented buffalo samples showed much lower numbers - 0.50% in 3% and 0.38% in 5% ones.

The ratio C18:2/C18:3 varied from 1.89 in control to 2.04 in 3% and 0.78 in 5% fortified yogurts and corresponds with contemporary recommendation for healthy nutrition (Oscarsson & Hurt-Camejo, 2017).

Table 2. Fatty acid content in plain and aronia supplemented yogurt, %

Fatty acids, %	Control	Aronia 3%	Aronia 5%
	Mean±SEM	Mean±SEM	Mean±SEM
C4:0	1.90±0.089	2.40±0.075	1.89±0.015
C6:0	1.53±0.037	2.41±0.095	1.20±0.033
C8:0	1.05±0.007	1.23±0.055	0.77±0.058
C10:0	2.86±0.012	3.19±0.005	2.43±0.069
C12:0	3.00±0.017	3.55±0.075	2.99±0.011
C13:0	0.17±0.015	0.21±0.003	0.21±0.015
C14:0	10.83±0.033	10.72±0.705	11.05±0.298
C14:1	0.62±0.005	0.87±0.005	0.72±0.057
C14:2	0.30±0.004	0.37±0.005	0.36±0.001
C15 iso	0.60±0.009	0.72±0.005	0.71±0.044
C15:0	1.29±0. 002	1.49±0.020	1.35±0.073
C16:0	29.46±0.110	27.70±0.645	29.54±0.529
C16:1	1.36±0.009	1.56±0.090	1.77±0.098
C16:2	0.51±0.001	0.36±0.005	0.47±0.001
C17:0	1.24±0.011	1.20±0.030	0.87±0.005
C17:1	0.25±0.006	0.25±0.005	0.29±0.001
C18iso	3.19±0.062	2.83±0.025	3.52±0.010
C18:0	9.60±0. 089	7.25±0.095	9.33±0.362
C18:1	26.53±0.752	27.17±1.025	27.29±0.346
C18:2	2.18±0.042	2.75±0.020	1.99±0.038
C18:3	1.15±0.062	1.35±0.015	1.04±0.009
CLA	0.57±0.002	0.50±0.008	0.38±0.009
SFA	66.73±0.971	64.85±1.220	65.81±0.199
UFA	33.27±0.971	35.15±1.220	34.19±0.199
MUFA	28.68±0.673	29.84±1.385	30.08±0.366
PUFA	4.60±0.011	5.32±0.065	4.12±0.049

*p>0.05 for all samples; SFA – saturated fatty acids; USA - unsaturated fatty acids;
MUFA - monounsaturated fatty acids; polyunsaturated fatty acids (PUFA); CLA - conjugated linoleic acid

Mineral content

The highest amounts of calcium, zink and copper were measured in raw milk. The percentage of phosphorus and potassium moved slightly down in control and in 3% aronia yogurt compared to the raw milk and in 5% samples raised, reaching the numbers of raw milk again (Table 3).

Table 3. Average mineral content in raw milk and in plain and aronia supplemented yogurts

Elements	Raw milk	Control yogurt	Aronia 3%	Aronia 5%
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Ca, %	0.200±0.000	0.199±0.000	0.190±0.003	0.188±0.001
P, %	0.156±0.000	0.151±0.001	0.138±0.002	0.151±0.003
K, mg/kg	1000±1.11	982±2.84	962±1.17	1004±10.31
Mg, mg/kg	192.9±1.13	189.2±9.81	192.3±8.65	177.2±6.27
Zn, mg/kg	5.66±0.040	5.25±0.045	5.20±0.318	5.28±0.044
Mn, mg/kg	0.118±0.002	0.122±0.001	0.129±0.002	0.104±0.003
Cu, mg/kg	0.416±0.006	0.388±0.130	0.382±0.012	0.351±0.008
Fe, mg/kg	1.73±0.020	1.85±0.012	1.80±0.010	1.71±0.021

Iron increased its amount in control and 3% samples and in 5% one decreased to the levels of raw milk. Significantly higher amount of iron was reported by Belyaev et al. (2022) in goat milk, supplemented with 5% aronia - 21.43 mg/kg, but lower of phosphorus - 0.109%. The quantity of calcium, magnesium manganese, zinc and copper gradually reduced and were lowest in 5% fortified yogurt. Our results differ from those of Paszczyk & Tonska (2022), where yogurts had higher contents of copper and manganese compared to the raw milk. Despite the fact, that aronia juice is rich source of potassium and zinc (Kulling & Rawel, 2008), there wasn`t considerable rise in their levels in fortified yogurts - on the contrary the percentage of zinc decreased with 0.38% in supplemented samples compared to the raw milk. The same was observed with the amounts of copper and manganese, even though, according to Pavlovic et al. (2015), aronia juice contains big amount of copper, and manganese. Decreased levels of these minerals can be explained with the metabolism of lactobacteria in the process of fermentation.

Vitamins

During our study, the levels of vitamin B1 (thiamine), B2 (riboflavin) and B6 (pyridoxin) were measured. All three vitamins showed in increase in their levels, most prominent in B6, which amount raised more than 3 times in 5% aronia samples compared to the control (Table 4).

Table 4. The content of vitamins B1, B2 and B6 in plain and aronia supplemented yogurts, mg/100 g

Sample	Vit. B1	Vit. B2	Vit.B6
	Mean±SEM	**Mean±SEM**	**Mean±SEM**
Control	0.061±0.001	0.098±0.002	0.051±0.001
Aronia 3%	0.058±0.001	0.146±0.004	0.057±0.001
Aronia 5%	0.069±0.003	0.162±0.003	0.166±0.003

Vitamin B1 is an essential cofactor in the metabolism of carbohydrates and amino acids (Smith et al., 2021). Its levels changed most insignificantly in the three types of yogurts. Vitamin B2 acts as an antioxidant (Cheung et al., 2014), plays an important role in regulation of cellular functions of and its derivatives are cofactors in essential enzyme-catalyzed reactions (Mosegaard et al., 2020). The quantity of riboflavin raised from 0.098 mg/100 g in control to 0.162 mg/100 g in 5% supplemented yogurt. Vitamin B6 has an important role in the function of the human nervous system and coenzyme forms of pyridoxine are involved in numerous biochemical reactions

(Dakshinamurti et al., 1990). Its amount was more than 3 times higher in 5% aronia yogurt compared to the plain one. The fruits of aronia are not a rich source of B1, B2, and B6 vitamins (Blinnikova, 2013) and this considerable raise of riboflavin and pyridoxine in 5% enriched samples may occur because of aronia`s juice bioactive components, that support the growth of LAB. Although some strains in starters are able to produce B vitamins (Gahriue et al., 2015), *Streptococcus thermophilus* and/or *Lactobacillus delbrueckii* subsp. *Bulgaricus* are not documented to have high capability to biosynthesize vitamins B2 and B6 (Patel et al., 2013). Presumably, the cooperation between mentioned ingredients resulted in increased formation of these vitamins in aronia yogurt.

Antioxidant activity

Yogurt in combination with fruits has a functional role in the human body due to the supply of fibre, vitamins, minerals, phytonutrients, polyphenols, anthocyanins (Limei et al., 2007). Fruit supplementing in yogurt is a popular approach to increase phenolic content and improve antioxidant profile. Also, yogurt fortified by natural antioxidants meets consumer requirements for "clean label" foods (Tolic, 2015; Gao et al., 2015).

Some fruits, e.g. Aronia are good source of phenolic compounds, especially of anthocyanins. Polyphenols are known to interact with milk proteins and form insoluble complexes. This reduces the total content of free polyphenols (Chouchouli et al., 2013).

In Table 5 are presented the results total flavonoid content (TFC) and the radical scavenging capacity obtained in yoghurt fortified by different amounts of Aronia juice. Yoghurt with 5% Aronia juice had the highest TFC (0.369 µg CE/L), and the control yoghurt - the lowest (0.075 µg CE/L). The total flavonoid content increased 2,3-, and 4,9-fold in the yoghurt with 3% and 5% aronia juice, respectively. The results in the present study confirmed those obtained by Nguyen & Hwang (2016), and Cuşmenco & Bulgaru (2020). The cited researchers concluded a proportional relationship between the TFC and the amount of juice added.

Table 5. The antioxidant activity of plain and aronia supplemented yogurt

Sample	Antioxidant activity	
	µmol TE/L	µg CE/L
Control	5.5	0.075
Aronia 3%	9.6	0.173
Aronia 5%	13.15	0.369
Aronia juice	508.2	220.9

Our research team found the same correlation between the radical scavenging capacity and the amount of aronia juice added. (Table 5): the lowest results are 5.5 µmol TE/L (control yoghurt sample) and the highest - 13.15 µmol TE/L (yoghurt with 5% aronia juice). This is expected, because the flavonoids are known with their antioxidant properties and especially with the ability to scavenge free radicals. Also, the higher antioxidant activity in yogurt containing aronia may result from the phytochemical content of the juice and microbial metabolic activity (Nguyen & Hwang, 2016).

CONCLUSIONS

The present study demonstrated that supplementation of buffalo yogurt with aronia juice effectively increased total amount of amino acids, including essential ones, the percentage of linolenic acid, of vitamins B2 and B6, the total flavonoid content and antioxidant activity in experimental samples. This contributed to buffalo`s yogurt high nutritional value and mineral content and opens up a possibility to add extra functionality to this type of product.

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IMTA KEY CONCEPT FOR DEVELOPING A STRATEGY TO INCREASE AQUACULTURE PRODUCTION AND IMPROVE ENVIRONMENTAL SUSTAINABILITY

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Abstract

This paper is a comprehensive study of the strategic approach, in the context of global population growth, to create more food using current natural resources. Integrated multi-trophic aquaculture (IMTA) can provide aquaculture products in much larger quantities using the same resources involved. Harnessing all the waste in the waters for this purpose brings multiple environmental and economic benefits. In the context of the circular economy, IMTA principles aim to significantly reduce waste and degrade the environment, but without restricting economic and social progress. In Romania, polyculture fish farming is practised to exploit all aquatic resources in fish farms. Each fish species is selected so that it can be nutritionally supported with natural feed from the environment, and various types of feed can be used to increase production. The results of the study can be used to improve the aquaculture development strategy, in the environmental sustainability context.

Key words: *aquaculture products, multi-trophic aquaculture, resource valorisation, sustainability, trophic level.*

INTRODUCTION

Integrated multi-trophic aquaculture (IMTA) is a similar practice to classic polyculture, in which the nutritional requirement is higher for all trophic levels and the use of feed is at full capacity. Aquaponics (the combination of aquaculture and hydroponics), fractional aquaculture, aquaculture and integrated systems, integrated peri-urban aquaculture systems and integrated fisheries systems are all variants of the key IMTA totalitarian concept. In this type of aquaculture there is an improvement in the efficiency of the growth of several aquatic species and a reduction of waste in the environment. Faecal matter from the upper trophic level is used by the lower trophic levels to increase the resource use efficiency of the rearing tanks. Lower trophic species can provide additional income while bringing multiple benefits to current practices in the sector. Making the most of the nutrient sources in the environment contributes to increasing its sustainability and creating new concerns in the field. The first published account of aquaculture dates back to 475 B.C. (Borgese, 1980). In 1970, John Ryther, considered the grandfather of integrated multi-trophic aquaculture (Chopin, 2013), together with his collaborators, developed modern mariculture, which is still practiced today in an integrated and intensive way. They demonstrated, both theoretically and experimentally, the integrated use of extractive organisms crustaceans, microalgae and seaweed in the treatment of domestic effluents, descriptively and with quantitative results. A source of used it was a domestic which was mixed with seawater, being considered as the nutrient source for phytoplankton, which in turn became food for clams and oysters (Rabanal, 1988). Nutrient wastes dissolved in the final effluent were filtered through biofilters from seaweeds (mainly Gracilaria and Ulva).

Globally, integrated multi-trophic aquaculture is the focus of extensive research to improve current practices in the industry and help develop a plan to increase production with a minimal environmental footprint. Increasing profitability in the context of this type of aquaculture implies bringing together more knowledge and setting the most environmentally friendly trajectory possible. For farmers involved in aquaculture this phenomenon brings multiple benefits with the valorisation of all resources and by-products resulting from the main fish farming process. Lower trophic chains can in turn use the excretion products from the main activity carried out, while boosting profits and obtaining more aquaculture products.

The main drawbacks of current fish farming systems highlight the use of large amounts of supplementary feed and the use of a large volume of water resulting in a large amount of externalised waste. In order to eliminate or reduce these disadvantages, a multilateral approach to the problems presented is needed, with the creation of new vectors capable of taking economic activity to another level. These vectors are under the scrutiny of many global organisations working to preserve and enhance environmental sustainability.

With alarming global population growth, the demands of providing the food needed to sustain this expansion are increasingly difficult to meet. With the global population growth, there is practically a shrinking of agricultural land and thus a reduction in the quantity of agri-food products. In order to compensate for this phenomenon, the active involvement of all resources to stabilise the balance is essential.

The strategic objectives aim to achieve more food per unit of decreasing area.

Integrated multi-trophic aquaculture emphasises the valuable qualities of algae by the way they purify water and thus ensure the health of aquatic ecosystems. Their ability to extract valuable components from the debris released into the pond by the first trophic chain is an important characteristic to be studied in detail.

In order to eradicate as much as possible the problems faced by European aquaculture such as: causing ecological damage and co-dependence on commercial feed, but also ensuring economic stability IMTA is an advantageous solution (Klinger & Naylor, 2012; Chopin et al., 2013; Granada et al., 2015; Hughes & Black, 2016).

MATERIALS AND METHODS

This study is a synthesis of the most comprehensive articles, which focus on the implementation of the key concept, of integrated multi-trophic aquaculture and its strengths/weaknesses. How the implementation of the IMTA key concept improves fish production and stimulates farmers in its application are some of the preliminary advantages. The development of a schematic analysis, of the main advantages following this concept, highlights the strengths of developing such a technology to increase productivity and reduce the environmental footprint.

RESULTS AND DISCUSSIONS

The most common aquaculture practice at national level is polyculture fish farming, but in other countries with intense concerns about the aquaculture sector, new approaches to this concept are being sought and the possibilities of combining different categories of life to create a symbiosis between them are being broadened. The establishment of such relationships is in some cases more difficult to control due to the particularities of each species involved. As the IMTA concept continues to change, it is important that all sectors involved in this industry are aware of the consequences of the changes involved so that they can adapt quickly and in an organised way. In order for research to move from 'pilot' to 'scale-up', some current regulations and policies may need to be changed, otherwise they will be seen as obstacles by industry partners, who will see no incentive to develop IMTA. This concept promotes economic and environmental sustainability through product transformation by-products and feed not consumed by organisms fed on intensive crops, thereby reducing eutrophication and increasing economic diversification (Bardach et al., 1972). Properly managed multi-trophic aquaculture accelerates productivity growth without harmful side effects (Table 1).

Improving productivity and reducing environmental impact according to (Wang el al., 2012), the production of one tonne of salmon results in a release of 50 kg of nitrogen into the environment, which could support the farming of 10 tons of algae or 5 tons of mussels over the entire fish production cycle (Holdt & Edwards, 2014). By recirculating nitrogen back into the farming system, a reduction in lipid and protein requirements occurs, thus decreasing the industry's negative impact on the environment while making production more efficient.

Table 1. SWOT analysis for IMTA key concept implementation (original)

SWOT analysis	
<u>Strengths</u> Increased production yield; Lower specific consumption; Use of the same land for additional production; Recovery of waste from the main activity.	<u>Weaknesses</u> Overpopulation of water bodies; Uneven growth of stocked fish material; Excess development of aquatic biomass; Destabilisation of physical-chemical water parameters. Fish escape from cages in open waters
<u>Opportunities</u> Increased production capacity; Increased numbers of living organisms and cultivated plants; Capture a larger sector of the market.	<u>Threats</u> Lack of knowledge about certain phenomena that occur gradually in production; Involvement of additional costs not foreseen in the initial financial analysis; Lack of knowledgeable staff.

The two types of wastes of interest are dissolved nutrients and organic materials such as: faecal matter and forage (in any form) not fully consumed (Sanderson et al., 2008). Trophic nutrient transfer, considered an ecological benefit, must occur in the proximity of farms; hence the potential for bioremediation in this perimeter is limited for algae and all sub-consumers of the intermediate chain (Broch et al., 2013; Cranford et al., 2013). Integrated Multi-Trophic Aquaculture (IMTA) is one of the modern aquaculture practices using offshore fish cages that are similar around the world (Figure 1). The design and degree of automation can change depending on the production, with the exception of floating closed containment schemes (Partridge et al., 2006; Fredriksson et al., 2008), most marine fish cages are operated as continuous flow rearing systems with floating nets.

Over time, the advent of "organic aquaculture" licences in countries such as Norway and Denmark has sparked a growing interest in obtaining such accreditations. At the same time, the increasing profitability of the sector, with decreasing environmental emissions, is stimulating individual operators to take new steps to align themselves with this strategy. In this way, extractive aquaculture produces a good amount of biomass, while providing the existing aquatic ecosystem with biomitical services for the surrounding ecosystem.

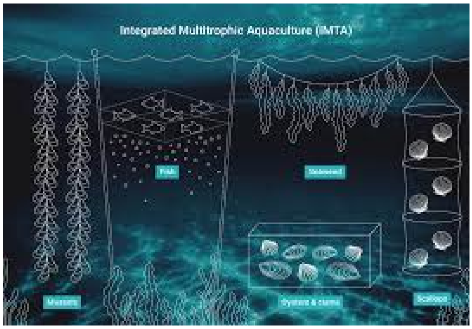

Figure 1. Schematic view of IMTA suitable marine species (Source: Magazine, 2022)

Bioremediation through IMTA in countries such as China to minimize the impact of nutrient losses from land to coastal waters has been remedied by large-scale cultivation of various species of seaweed, which subsequently succeeded in reducing nitrogen levels in the environment, successfully controlled phytoplankton spawning, and limited occurrence of toxic algal species (Xiao et al., 2017). Through multi-trophic aquaculture, a quantity of uneaten feed and waste, nutrients and by-products, considered "lost" from the feed, are recovered and transformed into feed that is easy to reintroduce into the production cycle and healthy, healthy and commercially valuable seafood through the removal of a partial amount of nutrients and CO , and the subsequent supply of O_2-oxygen. IMTA compliance can provide the producer with the economic security they need, even when production conditions are unfavourable (Whitmarsh et al., 2006; Ridler et al., 2007). Asia, supports more than two-thirds of the world's aquaculture production, IMTA was practiced here through trial and error, and experimentation many centuries ago (Sifa, 1987).

Interestingly, the civilisations that have made the most progress in developing integrated aquaculture systems regard waste as a valuable resource and have long since integrated the nutrients resulting from the first feeding stage. (Ling, 1977).

Efficient combinations of plants and aquatic organisms

Much attention has recently been focused on the specific interconnection between aquatic animals and algae in the water source. The most common combinations are those in which fish are the main link in the food chain, with other types of consumers such as crayfish, clams, in the case of seawater use shrimps and other crustacean species being introduced into the system in varying proportions. The combined use of these species makes it easier to obtain a greater quantity of product per unit area and to reduce losses. Integrated multi-trophic aquaculture, mainly based on the integrated selective culture of several fed aquatic species, such as fish, inorganic extractive species, (marine algae), and organic extractive species, promises to contribute to the sustainability of aquaculture (Chopin et al., 2002). The IMTA key concept is highly flexible. It can be applied in open water or terrestrial systems, in marine or freshwater systems (sometimes referred to as 'aquaponics') and in temperate or tropical systems (Figure 2).

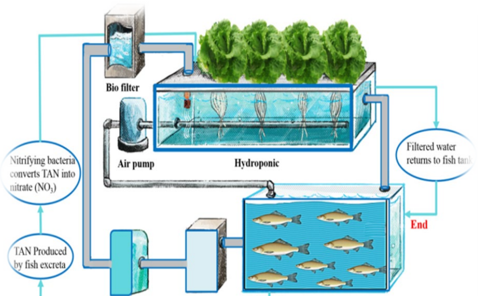

Figure 2. Aquaponics system diagram
(after Taha et al., 2022)

Significantly, suitable organisms are picked at different trophic levels based on their complementarity, their complementary roles in the biosystem and their ecological value or economic potential. In fact, IMTA does nothing more than recreate a simplified ecosystem that is in harmony with its environment, instead of introducing a single type of active biomass that is thought to be easily cultivated in isolation from all other aquatic organisms.

Integration as a concept should be understood as growing in proximity, not taking into account absolute distances, but rather connectivity in terms of ecosystem functionalities in the environment (Chopin et al., 2013). The viability of seaweed production is studied in both terrestrial and freshwater cultures, confirming that the nutrients delivered by supplementally fed aquatic life species are suitable for the growth of economically important seaweeds (Troell et al., 1999). The growth and rearing performance of seaweed is largely influenced by the appropriate selection of fish species, the design of the farm in which the experiment is conducted, feeding practices and other activity-specific parameters. The current economic situation and the factors related to it also differ from place to place. Therefore, the development of each farm applying the IMTA concept should aim to optimise for each individual aquaculture site. Organic effluents in whatever form they occur, resulting in concentrated forms from aquaculture farms can induced a series of negative effects with environmental impacts at the local level and can cause oxygen disturbance, eutrophication, changes in the biodiversity ecosystems and pollution of the water sources (Troell et al., 2003).

Population forms for growing fisheries taking into account the IMTA concept and introducing new consumer categories

Different fish farming technologies are used in fish farming depending on the type of fish farm and the purpose of the farmer. Countries such as Japan, China, South Korea, Thailand, Vietnam, Bangladesh, Indonesia cultivate aquatic species in both marine, wild and freshwater environments. They produce fish, shellfish and algae together in: ponds, lagoons, bays, etc.

Fish farming technologies are conditioned by: length of the growing season, management of existing fish stocks, use of fertilisers - fertilisation, supplementary feeding and production planning.

Many types of aquatic ponds can be successfully used for this method of fish farming: ponds, natural or artificial lakes, various water accumulations, canals or even rivers. As far as aquaculture production is concerned, it has been intensified by introducing more species into the system, which can cause a number of negative effects, such as degradation of water and soil quality in aquaculture ponds, stress and weak fish performance, a reduced profitability margin and overall environmental decline. In view of this, IMTA has proven the potential for greater crop diversity than a conventional commercial crop by introducing different consumers, such as molluscs and plants that can grow in water, such as water spinach, into the same pond (Kibria & Haque, 2018). Systems include agricultural/agricultural practices such as rice-fish and rice-crab, including the use of rice and crab to restore unproductive land to production in Inner Mongolia utilizing a rice variety that tolerates salt rates of up to 6 ppm. The diversification of production has significantly increased pond productivity while keeping water purity parameters at equilibrium levels in line with IMTA guidelines. This implies that IMTA could be advocated as effective for potential farmers to support food security for the poor. At S.C.D.P Nucet, the sturgeon *Polyodon spathula* was acclimatized and research was conducted on its feeding spectrum. (Costache et al., 2017). Therefore it can be introduced in ponds together with other planktonic fishes to exploit all the nutritive substrates. It can be introduced in ponds of 1 ha such as: 1000/sp carp, silver carp 500/sp, Polyodon 150/sp, raptors 200/sp. (Costache et al., 2020). The main food type of this fish is zooplankton. Therefore, it can be introduced into ponds together with other planktophagous fishes in order to exploit all the nutritive substrates.

Expanding production by growing different types of plants and molluscs

Freshwater snails eat organic detritus, seaweed and zooplankton, which in turn provide food for many fish species, birds and people. Snails are bioindicators and play a crucial role in the cleaning of water bodies, as they are saprophytes organism. Organic waste from unconsumed fish food in turn accumulates at the bottom of ponds and is used by snails as a food resource. Crustaceans take up intermediate trophic levels and sometimes play a dual function, both by filtering organics from the bottom and by generating ammonia. Food waste can also deliver additional nutrients; sometimes through direct ingestion, sometimes through breakdown as individual nutrients. In addition projects, nutrient residues are also collected and recycled into feed for captive fish. This can be done by processing cultured algae as feed. Water spinach is a very popular vegetable in Bangladesh for human food - all edible parts, except the submerged down parts, of the water spinach plant are edible. Water spinach thrives well in freshwater ponds throughout the whole year in tropical areas of the planet and needs sufficient nutrients, especially nitrogen. As observations from ponds where the IMTA concept was applied show, the growth of water spinach roots was of medium size; they did not reach the bottom of the water body but absorbed nutrients from the surrounding environment under immersed circumstances. This also showed that water spinach displayed a bio-mitigation effect and maintained nitrogenous residue concentrations in appropriate limits.

There is great uncertainty about climate change, the stability of aquatic ecosystems, fluctuating international markets and the preferences of consumers and other stakeholders. However, nutrient and by-product recirculation in aquaculture will certainly play a valuable role in increasing specific yields of global production. One of the principal benefits of filter feeding bodies such as Porifera is that their primary source of power is provided by super-efficient purification of organic particles ranging in size from 0.1 to 50 μm, such as suspended organic particles, eukaryotes and heterotrophs, phytoplankton and so on, such that their simple increase results in bioremediation of the surrounding environment. As a result, they are viewed as a possible way to reduce eutrophication and bacterial stock (Joseba et al., 2023). Water quality parameters are very important for aquaculture and depend on management issues such as stocking density, stocking material cultured organisms and supplements applied etc.

CONCLUSIONS

Hunger and unbalanced diets are still some of the most bitter problems facing humanity. In addition, there are growing concerns about the long-term viability of so many food production regimes, even fisheries and aquaculture, to meet future growing global supply demands. In this respect, implementing new, environmentally friendly practices that are friendly to institutions with such concerns are among the most readily available solutions of the moment. IMTA is essentially about matching needs with trade-offs to achieve a favourable long-term outcome of the food crisis.

In addition to the many advantages of the IMTA key concept, there are also some disadvantages of this practice. The costs of implementing such a system are often relatively high in the beginning, and the lack of knowledge on the subject is a real impediment. In addition to the two disadvantages mentioned above, in the case of combining several activities, there is the likelihood of overpopulation of the water body and its saturation.

Poor management of feed in the aquatic environment can also lead to excess formation of secretion products, which are reflected in an increase of organic matter in the water and hydrogen sulphide levels above normal limits. Solving these problems implies costs, which have ramifications for both the consumer market and aquaculture producers.

The circular economy can allow the aquaculture sector to grow and contribute by improving food products to increase as much as possible the timeframe in which resource depletion does not occur.

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BIODIVERSITY OF THE HELMINTH COMMUNITIES OF Carassius gibelio (Bloch, 1782) FROM MARITSA RIVER, BULGARIA

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Abstract

This study is the first to reveal the helminth fauna and helminth community's structure of Prussian carp (Carassius gibelio) from Maritsa River, Bulgaria. In 2022, ten Prussian carp specimens were collected from the Maritsa River and examined for parasites. 90% of the studied hosts harbour parasites. Two species of helminths were fixed: one from class Acanthocephala (Acanthocephalus anguillae Muller, 1780) and one from class Nematoda (Pseudocapillaria tomentosa Dujardin, 1843). The established nematode species is distinguished with high prevalence. Carassius gibelio is reported for the first time as a host for Pseudocapillaria tomentosa in Bulgaria. The river ecosystem of Maritsa is a new locality record for Pseudocapillaria tomentosa in Bulgaria.

Key words: *bioindication; Carassius gibelio; Maritsa River; helminth communities.*

INTRODUCTION

River Maritsa, whose length is 539 km, is the longest river on Balkan Peninsula. Its length on Bulgarian territory is 321.6 km, which makes it the fourth in length after the Danube, Iskar and Tundja. Maritsa springs from Rila Mountains at 2378 m altitude. The river has about one hundred significant tributaries, with the number of left and right tributaries almost equal. After crossing the border, the river passes through the territory of Greece and Turkey and flows into the Aegean Sea. The river's upper course covers the part from the source to the town of Belovo. The river's middle course covers the section through the Upper Thracian lowland from Belovo to the Bulgarian border. The lower course of Maritsa covers the section from the Bulgarian border to the mouth of the river. Almost the entire middle course of the river has been declared a protected area BG 0000578 "River Maritsa" according to Directive 92/43 and protected area BG 0002081 "Maritsa - Parvomaj" according to Directive 79/409.
The biodiversity of fish helminth communities from the Maritsa River was subject to investigation (Margaritov, 1965; Kirin, 2000a; Kirin, 2000b; Kirin, 2006; Kirin, 2013; Kirin, 2014; Chunchukova et al., 2019a; 2019b; Kuzmanova et al., 2019), endohelminth species biodiversity of *Carassius gibelio* is not explicitly examined. This study is the first that reveals the Prussian carp's endohelminth species biodiversity of from the river ecosystem of Maritsa, Bulgaria.

MATERIALS AND METHODS

In the summer of 2022, ten Prussian carp were caught from the Maritsa River in Plovdiv and examined for parasites. Plovdiv is located in the western part of the Upper Thracian lowland in Bulgaria and on both banks of the Maritsa River.
The width of the river section in this territory varies from 100 to 600 m, and the river's slope in the city area is minimal. On the part of Plovdiv, the rivers Parvenetska and Pyaschnik flow into Maritsa.
The fish was caught with a fishing line. The common and taxonomic name of fish is used in accordance with Fröse & Pauly (2022).
An incomplete parasitological examination was carried out immediately after their catch for helminths. Acanthocephalan specimens are examined in ethanol-glycerin as temporary slides and identified (Petrochenko, 1956; Ergens & Lom, 1970; Bykhovskaya-Pavlovskaya, 1985). The specimens from class Nematoda are studied in glycerine as temporary

slides and identified (Moravec, 2013). The helminths were determined following the keys of Bauer (1987).
The ecological terms mean intensity (MI), mean abundance (MA) and prevalence (P %) are used and calculated based on Bush et al. (1997). Based on the prevalence as suggested by Kennedy (1993), the parasites are grouped as core (>20), component (< 20) and accidental (< 10).

RESULTS AND DISCUSSIONS

During 2022, ten Prussian carp were caught from the river ecosystem of Maritsa and examined for parasites. *Carassius gibelio* (Pisces: Cyprinidae) is untypical and introduced species for aquatic ecosystems in Bulgaria (Stefanov, 2007). The Prussian carp is estimated as LC = Least Concern species (IUCN). *Carassius gibelio is* a freshwater, brackish, benthopelagic, potamodromous, omnivorous fish species (Fröse & Pauly, 2022). This cyprinid is distinguished by its rapid growth and its unpretentiousness to environmental conditions (Karapetkova & Zhivkov, 2010). The Prussian carp is tolerant of pollution and low oxygen levels (Kottelat & Freyhof, 2007). *Carassius gibelio* is widely distributed in Topolnitsa, Luda Yana, Stryama and Chepinska (Kolev, 2020).
From the study, ten specimens of Prussian carp from the Maritsa River 90.0% (9 fish) harbour parasites (Table 1). Thirty-six specimens (p) were established from two parasite taxa–one from class Acanthocephala (*Acanthocephalus anguillae* Müller, 1780) and one from class Nematoda (*Pseudocapillaria tomentosa* (Dujardin, 1843)). Both established parasite species occurred as adults.

Table 1. Helminth species of *Carassius gibelio* (N - number of studied fish, n - number of infected fish)

Helminth species	N = 10					
	n	p	P%	MI±SD	MA±SD	Range
Acanthocephalus anguillae (Muller, 1780)	1	2	10.00	2.0 ± 0.0	0.2 ± 0.6	0-2
Pseudocapillaria tomentosa (Dujardin, 1843)	9	34	90.00	3.78 ± 2.48	3.4 ± 2.62	1-8

The roundworm *Pseudocapillaria tomentosa* is distinguished with a high prevalence P% = 90.0 which determined it a core parasite, while *A. anguillae* (P% = 10.0) is an accidental species for the Prussian carp (Table 1).
The development of *A. anguillae* takes place with the participation of an intermediate host, *Asellus aquatics* (Linnaeus, 1758) (Kakacheva-Avramova, 1983). This crustacean indicates α-mesosaprobity (Johnson et al., 1993).
Even though the definitive hosts for *A. anguillae* are cyprinid, salmonid, percid, etc. fish (Kakacheva-Avramova, 1983), the paratenic host *Lutra lutra* was also reported for Bulgaria (Dimitrova et al., 2008).
Only two cyprinid hosts have been reported from river Maritsa for *Acanthocephalus anguillae,* with an enormous time gap between the studied hosts (Table 2).
In Europe, definitive hosts for *P. tomentosa* are fish from the family Cyprinidae (Moravec, 2013). The life cycle of *P. tomentosa* is incompletely established, but under natural conditions, oligochaetes play significant importance as a source of *P. tomentosa* infection for fish (Moravec, 2013).

Table 2. Fish hosts of *A. anguillae* reported from the Maritsa River

Host	Year
Alburnus alburnus	1965*[1]
	1965*[2]
Squalius cephalus	2000*[3]
	2000*[4]

[1]Kakacheva-Avramova
[2]Margaritov
[3]Kirin (a)
[4]Kirin (b)

Until now, only three fish species (see Table 3) are reported from Bulgarian rivers as hosts of *Pseudocapillaria tomentosa,* all cyprinid fish. The records are from Struma, Iskar and Danube (Nachev & Sures, 2009; Davidova et al., 2011; Zaharieva & Kirin, 2020). River Maritsa is a new locality, and *C. gibelio* is a new host record for *P. tomentosa* in Bulgaria.

Table 3. List of hosts of *Pseudocapillaria tomentosa* in Bulgaria

Host	Locality	Authority
Barbus barbus	Danube River	Nachev & Sures (2009)
Rhodeus amarus	Struma River Iskar River	Davidova et al. (2011)
Chondrostoma nasus	Danube River	Zaharieva & Kirin (2020)

Of the examined fish specimens, 80% were infected with only one parasite, the taxa - *Pseudocapillaria tomentosa* (Table 4). With two helminth species infected, only one fish host (10.00%). The average species richness in infracommunity of *C. gibelio* is 1.00 ± 0.45 species.

The most significant number of helminth specimens per host was eight found in two fish hosts. The average abundance in the infracommunities of Prussian carp is 3.6 ± 2.5. The parasite communities of *C. gibelio* from the Maritsa River showed a low value of Brillouin diversity index HB = 0.23 (Table 4).

Table 4. Infracommunities of *Carassius gibelio*

	Number of endohelminth species				
Carassius gibelio	0	1	2	Mean ± SD	Range
	1	8	1	1.0 0.45	0-2

	Number of endohelminth specimens			
	Total number	Mean ± SD	Range	Brillouin's index HB
Carassius gibelio	36	3.6 ± 1.5	0-8	0.23

Fish parasite fauna from the Maritsa River was also studied in previous cases (Margaritov, 1965; Kirin, 2000a; Kirin, 2000b; Kirin, 2006; Kirin, 2013; Kirin, 2014; Chunchukova et al., 2019a; 2019b; Kuzmanova et al., 2019), but Prussian carp's endohelminth species biodiversity was not explicitly examined. This study first reveals the parasite fauna of *Carassius gibelio* from River Maritsa. Twenty-six parasite species of Prussian carp in Bulgaria have been identified, although it is not a frequently studied species (Table 5).

Table 5. Overview of the established parasites of *Carassius gibelio* in Bulgaria and their locality

Helminth species \ Authority	Margaritov (1959)	Margaritov (1964)	Margaritov (1966)	Kakacheva-Avramova (1977)	Grupcheva & Nedeva (1999)	Shukerova (2005)	Atanasov (2012)	Kirin & Chunchukova (2021)	Chunchukova & Kirin (2021)	Kirin & Chunchukova (2022)	This study
Paradilepis scolecina (Rudolphi, 1819)					√						
Dactylogyrus anchoratus (Dujardin, 1845)	√	√		√	√						
Dactylogyrus extensus Mueller & Van Cleave, 1932		√									
Dactylogyrus formosus Kulwiec, 1927		√									
Dactylogyrus intermedius Wegener, 1909					√						
Dactylogyrus minutus Kulwiec, 1927		√									
Dactylogyrus vastator Nybelin, 1924		√									
Dactylogyrus vistulae Prost, 1957					√						
Dactylogyrus wegeneri Kulwiec, 1927		√									
Diplostomum helveticum (Dubois, 1929)					√						

Diplostomum pseudospathaceum Niewiadomska, 1984							√				
Diplostomum rutili Razmashkin, 1969						√					
Ancyrocephalus sp. Creplin, 1839					√						
Gyrodactylus medius Kathariner, 1893		√									
Gyrodactylus shulmani Ling, 1962					√						
Gyrodactylus sprostonae Ling, 1962					√						
Urocleidus similis (Mueller, 1936)					√						
Paradiplozoon homoion (Bychowsky & Nagibina, 1959)					√						
Posthodiplostomum cuticola (Nordmann, 1832)						√					
Nicolla skrjabini (Iwanitzky, 1928) Dollfus, 1960										√	
Raphidascaris acus (Bloch, 1799), larvae						√					
Contracaecum microcephalum (Rudolphi,1809), larvae						√					
Contracaecum sp. (Rudolphi,1809), larvae										√	
Pseudocapillaria tomentosa (Dujardin, 1843)											√
Ligula intestinalis (Linnaeus, 1758), larvae								√			
Acanthocephalus anguillae (Müller, 1780)							√		√		√
Pomphorhynchus laevis (Zoega in Muller, 1776)			√	√	√		√	√		√	
Locality	Chelopeche ne	Batak Reservoir	Danube	Danube	Zrebchevo reservoir	Lake Srebarna	Danube	Panicheri reservoir	Tundja	Tundja	Maritsa

CONCLUSIONS

This study is the first that reveals the parasite fauna of Prussian carp from the river ecosystem of Maritsa. Two parasite species were identified: *Acanthocephalus anguillae* and *Pseudocapillaria tomentosa*. The established nematode species *P. tomentosa* is distinguished with high prevalence. *Carassius gibelio* from the ecosystem of Maritsa represents a new host record in Bulgaria. River Maritsa is a new locality for *P. tomentosa*. *Acanthocephalus anguillae* is reported for the helminth communities of *C. gibelio* from the Maritsa River for the first time.

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URBAN ICHTHYOFAUNA: BECAȘ RIVER CASE STUDY, CLUJ-NAPOCA (TRANSYLVANIA)

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Abstract

The monitoring of ichthyofauna from rivers and rivulets crossing urban areas is important in the context of preserving ecosystems under continuous anthropic pressures. In addition, new invasive species may be observed. This study aims to present current data on the ichthyofauna of Becaș River, which has its entire course in Cluj-Napoca, Romania. For this purpose, alpha diversity, LWRs, Fulton condition factor (K) and relative condition factor (Kn) were determined. In total, 1216 specimens were analyzed, classified into 13 species, from 8 families. The species with the highest abundance was Pseudorasbora parva (46.46%), and the species with the lowest abundance were Cyprinus carpio and Perca fluviatilis (0.16%). Regarding LWRs, the lowest value of the b coefficient was obtained for Rutilus rutilus (2.7651) and the highest value for Phoxinus phoxinus (4.0868). The highest value of K was obtained for C. carpio (1.815) and the lowest value for Cobitis elongatoides (0.5942). The Kn was between 0.5436 (Gobio carpathicus) and 1.0330 (C. elongatoides).

***Key words**: anthropization, aquatic ecosystem, Cyprinidae, invasive species.*

INTRODUCTION

Fish populations in natural waters are in a continuous dynamic in terms of species structure. The factors leading to these changes are numerous (Bănăduc et al., 2023). The most important are represented by anthropogenic influences that include changes in water courses (Hellström et al., 2019; Holban et al., 2020; Dănălache et al., 2020), expansion of urban habitats (Burger et al., 2022), hydrotechnical facilities (Piria et al., 2019), irrational exploitation of fish populations, poaching (Gotkiewicz, 2018), predatory species (Oehm et al., 2022) and pollution resulting from industrial, agricultural activities and the discharge of rainwater, wastewater and household water (Casatti et al., 2006; Jacobson, 2011). Agricultural activities that include the use of fertilizers, especially inorganic compounds such as nitrogen and phosphorus, have a primary effect on the development of phytoplankton communities (Kremser & Schung, 2002), which represent the primary food for the juveniles of various fish species. In principle, this is a favorable consequence, but various chemical products such as insecticides, pesticides, or herbicides (Gibbons et al., 2015; Houssou et al., 2018; Yang et al., 2021) can have long-term implications that will lead to degenerative aspects of fish populations, especially with regard to the reproductive and perpetuating functions of species, thus influencing aquatic biodiversity (Higgins et al., 2019; Jwaideh et al., 2022) and fish health. Domestic and industrial waters discharged into watercourses in urbanized areas can have a similar effect (McCallum et al., 2017).

Other factors responsible for the dispersion of fish species are geo-morphological and climatic factors (Georgiev & Nazarova, 2015; Bănăduc et al., 2022), their parameters changing continuously. Sometimes, even without the influence of the previously listed factors, structural changes occur generated by the ecological relationships between different species (Zeng et al., 2022), the accidental appearance of new fish species (Brysiewicz et al., 2022; Larentis et al., 2022), the overlapping of trophic niches (Leunda, 2010; Alonso et al.,

2019) or the uncontrolled numerical increase of specimens, which will lead to a drastic reduction of food resources. Last but not least, economic and commercial activities also contribute to changes in species structures (Cocan et al., 2014; Cocan et al., 2016).

Currently, there is an increasingly accentuated trend of migration of human populations from rural to urban environments. The reasons are complex, but generally relate to the availability of better living conditions, wage gaps between rural and urban environments (Stanef, 2014), easier access to jobs, education, and health services. These migrations lead to the spatial development of large cities, sometimes in homogeneously (Földes, 2019), including the expansion of areas intended for housing. Thus, the peripheral areas of cities that in the past were either agricultural areas or unexploited areas, become new neighborhoods that exert intense anthropogenic pressures on natural environments.

The expansion of urban areas can have catastrophic effects on fish populations in the rivers that cross these areas (Panja et al., 2020). However, there are situations in which fish species adapt to new conditions (Czeglédi et al., 2020), both in terms of the physicochemical parameters of the water and in terms of food resources (Peressin et al., 2018). Sometimes, the process of adaptation to new conditions can take several years (Sun et al., 2022). Moreover, previous studies and researchers' observations have demonstrated many times that river sectors in urbanized areas are more abundant with fish compared to isolated areas (Qiao et al., 2022). This is the result of easier access to food resources (Tófoli et al., 2013), generally represented by food waste dumped uncontrolled into watercourses by humans. Fish adapt to new food resources (Ganassin et al., 2019) and multiply numerically.

Fish populations in the urban areas of Romania have been scarcely studied both in terms of fish diversity and abundance. Thus, the purpose of this study is to determine the species structure and biodiversity indices of fish in the Becaș River, a tributary of the Someș-Tisa hydrographic catchment. This river crosses the city of Cluj-Napoca from South to North and it is subject to different anthropic pressures on its route from the sources to the outlet, from areas with real estate potential, to commercial and industrial areas, crossing, among others, the entire area belonging to the international airport.

MATERIALS AND METHODS

Fish sampling was conducted using a SAMUS 725 MP electrofishing apparatus, powered by a 12V (24A) rechargeable battery. Each fish was weighed (BW = fish wet body weight) and measured (TL = total length of fish) and data was used for length-weight relationships (LWRs), Fulton's condition factor (*K*), and relative condition factor (*K*n) (Reid et al., 2009; Imecs & Nagy, 2016; Cocan et al., 2020; Lațiu et al., 2023). Based on the number of encountered species and their frequencies, alpha diversity indices were determined.

Relative abundance (%), Simpson's index (1-D), Shannon's index (H'), Evenness (J'), Margalef index (Md) and Berger-Parker index (d) were determined to provide general information on species structure and to be used for future conservation plans for aquatic habitats (Cheng et al., 2019). Alpha diversity indices were determined using Past 4.03 software (Hammer & Harper, 2001; Hammer & Harper, 2006).

LWRs were calculated using the formula $BW = aTL^b$, where a and b are the coefficients of the regression between BW and TL (Le Cren, 1951). Coefficients a and b were obtained by the least-square linear regression from the log-transformed values of TL and BW, using the formula $BW = \log a + b \log TL$ (Morey et al., 2003). To determine the type of growth for the sampled specimens, b values were analyzed as follows: positive allometric growth, if $b>3$; negative allometric growth, if $b<3$; and isometric growth, if $b=3$ (Froese, 2006). Confidence intervals (CI) at 95% were used to establish if the b values obtained from the linear regressions were significantly different from the isometric value ($b = 3$). The t-test was used to determine if the obtained b value was significantly different from the isometric value and establish the growth type (Ricker, 1975; Zar, 1984).

Fulton's condition factor (K) was obtained using the formula $K = 100*BW/TL^3$, where K is the value of the index, BW is the fish's wet body weight and TL is the total length of the fish (Fulton, 1904; Ricker, 1975; Nash et al., 2006). The relative condition factor (*K*n) of each individual was determined by the following equation: $Kn = W_o/We$, where W_o is the observed/determined wet weight of the fish and W_e is the expected weight, determined from the LWRs (Narejo et al., 2002). The fish condition can be evaluated as follows: $Kn \geq 1$, when fish growth condition is good, and $Kn < 1$, when the fish growth condition is poor (Le Cren, 1951; Cone, 1989).

RESULTS AND DISCUSSIONS

Becaș River is a right-hand tributary of Someșul Mic River, being located in the eastern part of Cluj-Napoca City, flowing from South to North. This small river has a total length of 9 km and overpasses major infrastructures of the city such as industrial, commercial and transportation areas (Figure 1).

A total number of 1216 specimens from 13 species, belonging to 8 families (Centrarchidae, Cobitidae, Acheilognathidae, Cyprinidae, Gobionidae, Leuciscidae, Nemacheilidae, Percidae) were captured from 15th to 17th of June, 2022. The most abundant species was *Pseudorasbora parva* (Temminck and Schlegel, 1846), representing 46.46% of the total catch. Another highly abundant species was *Rutilus rutilus* (Linnaeus, 1758) representing 38.82%. *Barbatula barbatula* (Linnaeus, 1758) specimens represented 2.47% of the analyzed specimens. A similar percentage (2.38%) was observed in the case of *Squalius cephalus* (Linnaeus, 1758). *Phoxinus phoxinus* (Linnaeus, 1758) and *Cobitis elongatoides* (Băcescu & Mayer, 1969) had similar abundances, 0.99% and 0.90% respectively.

Figure 1. Riverine landscape across the studied area: A - Confluence of Becaș River and Someșul Mic River; B - Concrete banks from the Airport Area; C - Landscape from the Industrial Area of the River; D - Landscape from the Commercial Area of the River

The specimens of *Barbus carpathicus* (Kotlík et al., 2002) represented 0.82% of the total catch. The specimens of *Lepomis gibbosus* (Linnaeus, 1758) represented 0.41% of the catch. *Rhodeus amarus* (Bloch, 1782) represented 0.25% of the analyzed fishes. *Perca fluviatilis* Linnaeus, 1758 and *Cyprinus carpio* Linnaeus, 1758 had identical abundance (0.19%) (Figure 2).

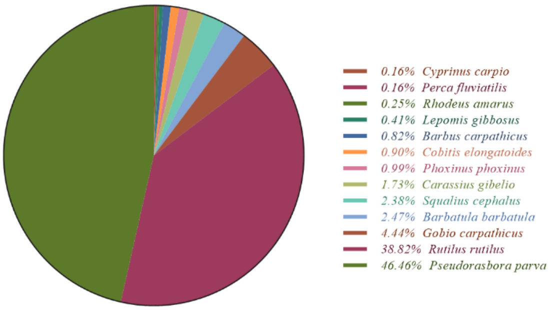

Figure 2. Relative abundance for fish species observed in Becaș River

Simpson's index 1-D showed a moderate degree of diversity (1-D = 0.6297). Shannon's index H', takes into consideration both species richness and evenness, showing low-moderate diversity (H' = 1.2818). In this case, the determined Evenness (J' = 0.5061) articulates the fact that Becaș river has a moderate evenness between the species. Regarding the Margalef index (Md), a moderate degree of diversity was observed, similar to Simpson's index. The Berger-Parker index (d), expressing, in general, the proportional importance of the most abundant species, showed that *P. parva* dominates the fish community (d = 0.4646) (Table 1.).

Table 1. Alpha Diversity Indices for the fish species observed in Becaș River

Species richness (number of species)	13
Number of individuals	1216
Simpson 1-D	0.6297
Shannon H'	1.2980
Evenness J'	0.5061
Margalef Md	1.6890
Berger-Parker d	0.4646

From the total of 13 species observed in Becaș river, 8 species showed positive allometric growth (*L. gibbosus*, *C. elongatoides*, *R. amarus*, *B. carpathicus*, *C. gibelio*, *G. carpathicus*, *P. parva*, *P. phoxinus*), 2 species showed isometric growth (*S. cephalus* and *B. barbatula*), 1 species showed negative allometric growth (*R. rutilus*) and for 2 species (*C. carpio* and *P. fluviatilis*) the LWRs were not determined due to the small number of specimens (Table 2).

Parameter/exponent *b* ranged from 2.7651 (*R. rutilus*) to 4.0868 (*P. phoxinus*). The unusual value observed in the case of *P. phoxinus* may be caused by the length class of the analyzed specimens and the small number of analyzed individuals. The coefficient of determination r^2 ranged from 0.8181 (*C. elongatoides*) to 0.9998 (*L. gibbosus*).

Fulton's condition factor *K* showed ununiform variations across the individuals and also across the species. In the case of *L. gibbosus*, the mean value of *K* was 1.680±0.0805 and a coefficient of variation (CV%) of 4.796%; for *C. elongatoides* *K*=0.5942±0.2024 and CV% = 34.06; for *R. amarus* mean *K* = 1.343±0.2463 and the CV%=18.34; for *B. carpathicus* mean *K* = 0.8538 ± 0.0967 and CV% = 11.33; for *C. carpio* mean *K* = 1.815±0.0368 and CV% = 2.030; for *C. gibelio* mean *K* = 1.651±0.1331 and CV% = 8.063; for *G. carpathicus* mean *K* = 0.8784 ± 0.1296 and CV% = 14.75; for *P. parva* mean *K* = 0.8076±0.1808 and CV% = 22.39; for *P. phoxinus* mean *K* = 0.7718 ± 0.2162 and CV%=28.01; for *S. cephalus* mean *K* = 0.9121 ± 0.0745 and CV% = 8.170; for *R. rutilus* mean *K* = 1.090 ± 0.2334 and CV% = 21.42; for *B. barbatula* mean *K* = 0.8265±0.0827 and CV% = 10.01; and for *P. fluviatilis* mean *K* = 1.305±0.0876 and CV% = 6.712 (Figure 3).

The mean relative condition factor *K*n ranged from 0.5436 ± 0.0778 in the case of *G. carpathicus* to 1.0330 ± 0.2991 in the case of *C. elongatoides* (Figure 4).

Table 2. The determined LWRs for fish species observed in Becaș River

Family	Species	n	Total length (mm)		Body weight (g)		Parameters of LWRs					Growth type
			TL min.-max.	TL mean ± SD	BW min.-max	BW mean ± SD	*a*	*b*	*a* CI95%	*b* CI95%	r^2	
Centrarchidae	*Lepomis gibbosus*	5	3.7-9.8	7.28 ±3.012	0.8-16.9	9.26 ±7.637	0.01421	3.0876	0.0119-0.0169	2.9981-3.1771	0.9998	b≠3 (ALO+)
Cobitidae	*Cobitis elongatoides*	11	6.5-9.8	8.055 ±1.16	1.4-9.8	3.445 ±2.436	0.00143	3.6667	0.00009-0.02181	2.35882-4.97466	0.8171	b≠3 (ALO+)
Acheilognathidae	*Rhodeus amarus*	3	3.8-6.1	5.2 ±1.229	0.6-3.6	2.233 ±1.518	0.00430	3.6931	0.00004-0.46904	0.08338-6.5524	0.9963	b≠3 (ALO+)
Cyprinidae	*Barbus carpathicus*	10	4.5-15.4	8.87 ±4.759	0.7-37.1	11.89 ±14.55	0.00606	3.1644	0.00490-0.00750	3.06363-3.26513	0.9985	b≠3 (ALO+)
	Cyprinus carpio	2	n too small									
	Carassius gibelio	21	3.8-14.6	8.814 ±3.785	0.8-50.3	17.7 ±16.88	0.01334	3.1012	0.01167-0.01526	3.03805-3.16437	0.9982	b≠3 (ALO+)
Gobionidae	*Gobio carpathicus*	54	3.2-12.8	7.056 ±2.726	0.3-19.6	4.761 ±5.241	0.00650	3.1534	0.00529-0.00799	3.04615-3.26072	0.9853	b≠3 (ALO+)
	Pseudorasbora parva	565	2.3-8.4	4.212 ±0.8682	0.1-6.6	0.7207 ±0.6288	0.00452	3.3920	0.0040-0.0052	3.2991-3.4849	0.9014	b≠3 (ALO+)
Leuciscidae	*Phoxinus phoxinus*	12	3.1-6.1	4.675 ±1.017	0.1-2.2	0.975 ±0.6717	0.00141	4.0868	0.0005-0.0044	3.3514-4.8222	0.9388	b≠3 (ALO+)
	Squalius cephalus	29	4.8-22.5	11.86 ±4.238	1-112	21.33 ±21.47	0.00789	3.0593	0.00659-0.00945	2.9851-3.1334	0.9962	b=3 (ISO)
	Rutilus rutilus	472	3.4-11.9	5.352 ±1.386	0.3-17.8	1.99 ±2.105	0.01570	2.7651	0.01376-0.01791	2.6859-2.8443	0.9093	b≠3 (ALO-)
Nemacheilidae	*Barbatula barbatula*	30	4.1-11.2	7.613 ±2.031	0.6-12.3	4.447 ±2.941	0.00675	3.0991	0.00519-0.00787	2.96856-3.22969	0.9883	b=3 (ISO)
Percidae	*Perca fluviatilis*	2	n too small									

Legend: n - number of individuals; TL - total length (mm); BW - wet body weight (g); SD - standard deviation; *a* and *b* - are the coefficients of the regression; CI 95% - confidence intervals; R^2 - coefficient of regression; ISO - isometric growth; ALLO+ - positive allometric growth; ALLO– - negative allometric growth.

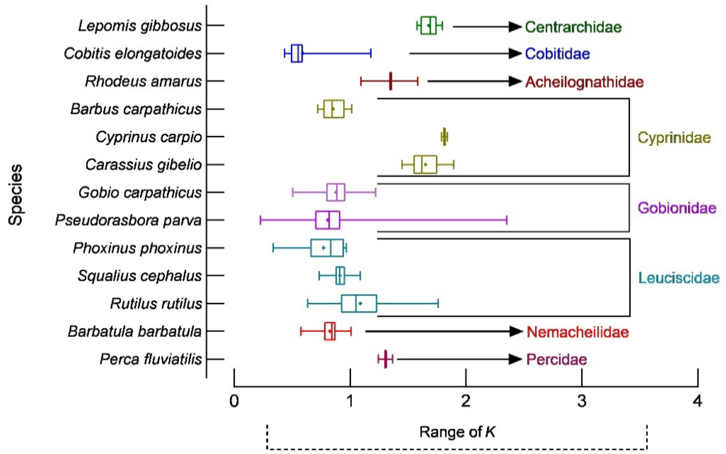

Figure 3. The determined Fulton Condition Factor (K) for the fish species found in Becaș River

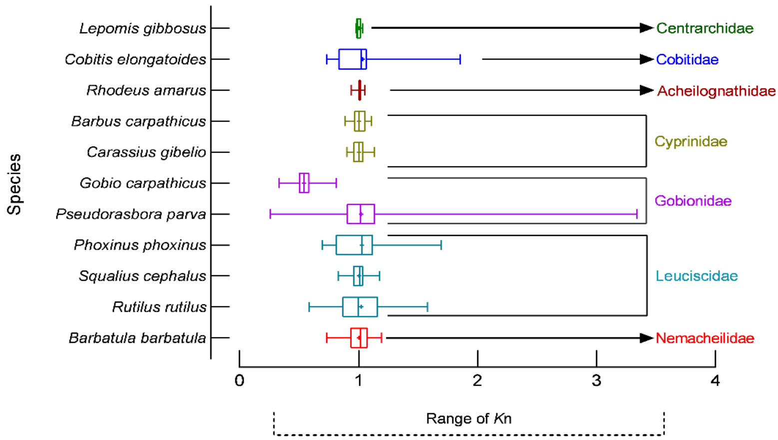

Figure 4. The determined Relative Condition Factor (Kn) for the fish species found in Becaș River

When analyzing the entire sampled population, it can be observed that only one species (*G. carpathicus*) presented the *K*n mean value smaller than 1, suggesting that its growth condition was poor. Two species (*L. gibbosus* and *R. amarus*) had *K*n equal to 1, suggesting that they were in normal growth conditions. The remaining species (*C. elongatoides, Barbus carpathicus, Carassius gibelio, P. parva, P. phoxinus, S. cephalus, R. rutilus* and *B. barbatula*) except *C. carpio* and *P. fluviatilis* (due to the small number of specimens) showed a mean *K*n value larger than 1, suggesting that the species were in good growth condition.

The determined LWRs were compared to the available data from FishBase (Froese & Pauly, 2000). Species such as *L. gibbosus, B. carpathicus, G. carpathicus, S. cephalus,* and *B. barbatula* had similar *b* exponent values compared to those provided by FishBase. Other species such as *C. elongatoides, R. amarus, C. gibelio, P. parva, and P. phoxinus* had larger *b* exponent values compared to those from Fishbase. Only one species (*R. rutilus*) had a smaller *b* exponent value. *C. carpio* and *P. fluviatilis* were not compared to the above-mentioned source, because of the small number of specimens. In terms of occurrence, 8 species (*C. elongatoides, R. amarus, C. gibelio,* *P. phoxinus, S. cephalus, R. rutilus, B. barbatula* and *P. fluviatilis*) are mentioned as native by FishBase. Three species (*L. gibbosus, C. carpio* and *P. parva*) are mentioned as introduced by the same data source. Two species (*B. carpathicus* and *G. carpathicus*) were not mentioned to be present in Romanian freshwater ecosystems by FishBase, but they were mentioned by other sources to be present in Romanian freshwater ecosystems (Kotlík et al., 2002; Konopiński et al., 2013; Năstase & Oțel, 2015; Ardelean et al., 2016). Iftimie & Iftimie (2021) mentioned two of the species observed in the present study as introduced/alien (*L. gibbosus* and *P. parva*), with unclear status under Romanian law, both potential threats to native fish species. According to the IUCN Red List, 11 of the observed species from the present study were under the "LC - Least Concern" category. *C. gibelio* is not mentioned and *C. carpio* is considered "VU - Vulnerable" (IUCN Red List, accessed November 15, 2022).

CONCLUSIONS

This is the first study on the ichthyofauna of the Becaș River, Cluj County, Romania. Although it is a small body of water, about 9 km long, it is home to 13 species of fish, of which 10 species are native and 3 are introduced. The length-weight relationship, the Fulton condition factor (*K*) and the relative condition factor (*K*n) show that the maintenance status of the fish population is generally good for most species. The continuous monitoring of the ichthyofauna of the Becaș River is necessary, because the composition of the species and their abundance can provide important information regarding the anthropogenic impact.

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THE GROWTH AND DEVELOPMENT OF KALE AND ARUGULA IN AN AQUAPONIC SYSTEM

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Abstract

The objective of the current study was to determine the effect of two fish stocking densities on the growth performance, development, and antioxidant capacity of kale (Brassica oleracea L. var. acephala) and arugula (Eruca vesicaria ssp. sativa) in an aquaponic system with common carp (Cyprinus carpio). The aquaponics system consists of six rearing units for fish and twelve units for plants, purple light-led lamps for plants (36 W), biological and mechanical filters, and pumps for water recirculation. Two fish stocking densities were used: 3.5 kg×m^{-3} *and 7 kg×m*$^{-3}$*, each replicated three times. For each treatment or fish stocking density, 15 kale seedlings (51 plants×m*$^{-2}$*) and 15 arugula seedlings (51 plants×m*$^{-2}$*) were planted. All treatments were done in triplicates. At the end of the trial, the fresh weight of the plants was measured, and the results showed that the stocking density of the common carp of 7 kg×m*$^{-3}$ *resulted in higher production of kale and arugula by maintaining good water quality for the plant and fish.*

Key words*: aquaponic, carp, plant production.*

INTRODUCTION

Aquaponic systems are a sustainable and efficient method of producing both plant and fish products in a closed-loop system. This system integrates hydroponic plant production with aquaculture, where the fish waste provides the necessary nutrients for the plants to grow, while the plants purify the water for the fish (Sfetcu et al., 2008; Filep et al., 2016a; 2016b; Atique et al., 2023). Aquaponics systems do not waste energy, water, or nutrients (Rizal et al., 2018; Jansen & Keesman, 2022). Lately, these systems have become increasingly popular, mainly because of the numerous advantages compared to conventional agriculture. Aquaponics used less water than traditional farming methods because the water is recirculated within the closed-loop system.

Among the various crops that can be grown in aquaponic systems, kale, and arugula are two leafy green vegetables that are known for their high nutritional value and antioxidant activity. Kale (*Brassica oleracea var. acephala*) and arugula (*Eruca sativa*) are both members of the *Brassicaceae* family (Sikora & Bodziarczyk, 2012). Kale is rich in sources of dietary fiber, with a high content of vitamins A, K, and C, minerals, such as potassium (K), calcium (Ca), and magnesium (Mg), and significant amounts of carotene and folate (Walker & Weinstein, 1994). Arugula (*Eruca vesicaria* ssp. *sativa*) is a cruciferous vegetable commonly known as rocket salad which contains high levels of beneficial nitrates and polyphenols, and high levels of antioxidants, such as vitamins C, K, A, and iron (Amorim et al., 2007).

In the last years, there was a growing interest in using aquaponic systems to grow these leafy greens because of their fast growth and relatively lower operation costs and due to their increasing popularity and demand in the health food industry. Also, a big advantage of the production of these vegetables in the aquaponic systems is a veritable way of earning a stable income because yield is possible year-round. Good performance results have been obtained for growing kale and tilapia in an integrated aqua-vegeculture (IAVC) system, and deep-water culture (DWC) system (pilot-scale evaluation), at a stocking density of tilapia of 10 kg/m^3, while the density of kale seedlings was 25 plants/m^2 (Afolabi, 2020). In contrast, Barbosa et al., 2020, studied the growth of

arugula under two different system water volumes (500-L vegetable tank: 500-L fish tank, and 500-L vegetable tank:1000-L fish tank), and their results indicated poor growth performance mainly due to the extreme high sowing density of seedlings (40 seedlings of arugula per growing unit, approx. 0.160 kg/m^2).
In this context, this study aimed to evaluate the growth of kale and arugula vegetables in an aquaponic system, using as substrate Light Expanded Clay Aggregate (LECA), together with common carp (*Cyprinus carpio* L., 1758), stocked at two densities (SD1-3.5 kg×m^{-3}, respectively SD2-7 kg×m^{-3}). Also, the results of the study may contribute to developing integrated and sustainable food production using modern methods.

MATERIALS AND METHODS

Experimental design. The study was conducted for 41 days at the Aquaponics station of the Research Center 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 system consists of six rearing units for fish and twelve rearing units for plants, led lamps with purple light (36 W), biological and mechanical filtration, and pumps for water recirculation. The aquaponics system was previously described in our research (Crețu et al., 2022). For the experiment, a total number of 90 common carp were used (*Cyprinus carpio* L, 1758), with an average initial weight of 116.62 grams. Two fish stocking densities were created: SD1-3.5 kg×m^{-3}, and SD2-7 kg×m^{-3}, each replicated three times (Figure 1). The plant units were populated with 15 kale seedlings (*Brassica oleracea* var. *acephala*), respectively 15 arugula seedlings (*Eruca sativa*) in each rearing unit (51 plants/m^2). The substrate used for the growth of kale and arugula was represented by Light Expanded Clay Aggregate (LECA). The high surface area of the substrate provides more space for the growth of nitrifying bacteria. The plants were obtained from seeds in our laboratory and then transferred into the aquaponic system at 21 days.

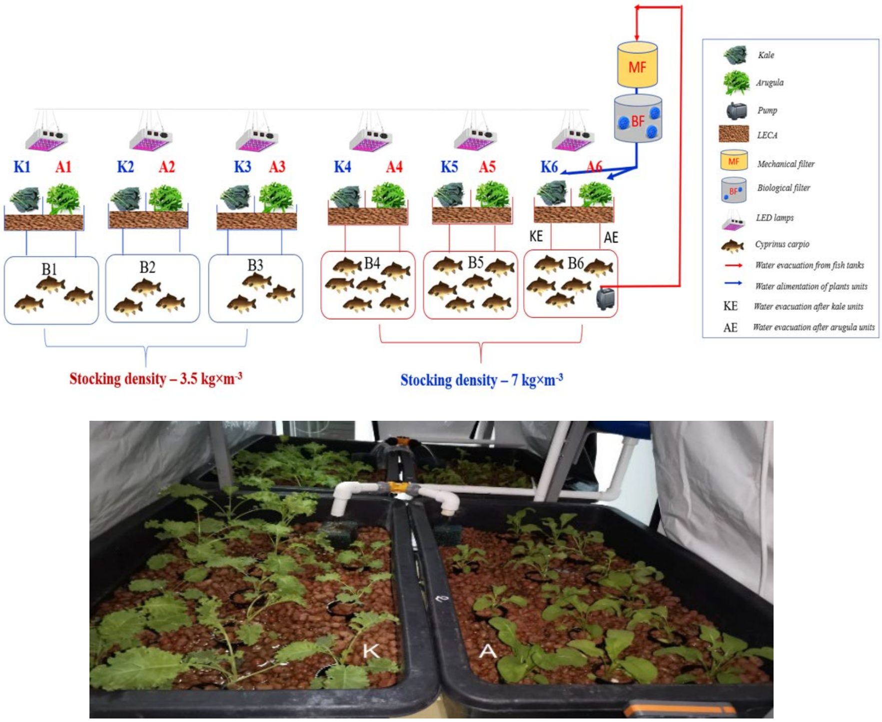

Figure 1. The scheme of the experimental design (K-kale, A-arugula)

During this trial, fish were fed two times a day at 08:00 a.m. and 4:00 p.m. at a rate of 2% of their body weight (BW), with a commercial diet (43% protein, 12% fat, 4% fiber, 6% ash).

Water quality. During the experimental period, the water quality parameters, such as dissolved oxygen, temperature, pH, and conductivity were measured daily with the help of a portable multiparameter Hanna HI98494 (Hanna Instruments, Cluj, Romania). The concentration of the nitrogen compounds ($N\text{-}NO_2^-$; $N\text{-}NO_3^-$, $N\text{-}NH_4^+$) was measured twice per week with the help of the Spectroquant Nova 400 photometer compatible with Merck kits (Merk Romania, Bucharest, Romania).

Fish and plant growth parameters. At the beginning of the experiment and the end, kale and thyme seedlings were measured for plant height (cm), plant weight (g), and roots height (cm). Fish growth parameters, including initial weight (g), final weight (g), weight gain (g), initial biomass (g), final biomass (g), feed conversion ratio (FCR), specific growth rate (SGR), and protein efficiency ratio (PER) were calculated:

- Weight Gain (WG) = Final Weight (Wt) (g) – Initial Weight (W0) (g);
- Food Conversion Ratio (FCR) = fish feed quantity (g)/WG (g) (g/g);
- Specific Growth Rate (SGR) = (ln Wt – ln W0)/t × 100 (% BW/day),
  where t - duration of the experiment;
- Protein efficiency ratio (PER) = Total weight gain (W)/amount of protein fed (g).

For the plant's evaluation, all plants were weight individually at day 0 (when plants were transferred into the aquaponic system) and at day 41 (when plants were removed from the aquaponic system).

Data analysis. All collected data were analyzed with the SPSS 21.0 (SPSS Company Inc., Chicago, IL. USA) statistical software package and Microsoft Excel. Statistically significant differences were reported at $p<0.05$.

RESULTS AND DISCUSSIONS

Water quality in the aquaponic system. In an aquaponic system, the physicochemical parameters played a significant role in obtaining a successful production. The water quality variables were maintained at adequate levels for the rearing of carp while providing the necessary nutrients for plant growth (Table 1). The temperature was in the optimum range for the growth of carp (Muralitharan & Dhanushsri, 2022), without any significant differences ($p>0.05$) between the growing units of carp and plants. Regarding the growth of plants, kale is a cool-season vegetable that grows best in temperatures between 15-18°C but can tolerate temperatures as 27°C degrees for short periods (Afolabi, 2020), while for optimal growth of arugula, the temperatures should be about 25°C (Furlani et al., 1999). The dissolved oxygen and pH values registered no significant ($p>0.05$) different values between the growing units of carp and plants. Regarding the values of the nitrogen compounds, significantly lower values ($p<0.05$) were recorded between the rearing units of carp and after the water evacuation from the growing units of Kale and arugula. The lowest values were recorded after the evacuation of water from the plant units.

Table 1. The physicochemical parameters of water during the experimental trial

Parameter	Fish tanks		Plant tanks				
	SD1	SD2	AAU	KE		AE	
				SD1	SD2	SD1	SD2
Temperature (°C)	21.2±0.10	21.4±0.09	21.30±0.10	21.4±0.09	21.09±0.11	21.4±0.08	21.8±0.10
Oxygen ($mg \times L^{-1}$)	7.21±0.76	7.62±0.64	7.18±0.10	7.16±0.10	7.26±0.11	7.41±0.12	7.36±0.09
pH (pH units)	7.68±0.11	7.61±0.12	7.65±0.19	7.39±0.14	7.46±0.16	7.36±0.12	7.26±0.11
$N\text{-}NO_2^-$ ($mg \times L^{-1}$)	0.04±0.013	0.05±0.011	0.03±0.013	0.01±0.011	0.02±0.011	0.01±0.016	0.03±0.013
$N\text{-}NO_3^-$ ($mg \times L^{-1}$)	27.80±4.52	32.4±8.12	18.63±3.12	12.10±4.52	16.09±6.53	11.56±6.23	9.24±9.45
$N\text{-}NH_4^+$ ($mg \times L^{-1}$)	0.47±0.17	0.61±0.78	0.46±0.15	0.25±0.11	0.33±0.19	0.29±0.26	0.44±0.16
Conductivity (μS/cm)	1923±65.50	1931±89.62	1896±89.16	1889±75.16	1894±76.14	1923±85.13	1965±85.45

Note: AAU- alimentation of the aquaponic units (after the evacuation of mechanical and biological filtration); KE- water evacuation after growing units of Kale; AE- water evacuation after growing units of arugula; Fish tanks- SD1 - the values are presented as the mean±SD of the B1, B2, and B3 1tanks; SD2- the values are presented as the mean values of the B4, B5, and B6 tanks; Plant tanks- KE- SD1 - the values are presented as the mean±SD of the K1, K2, and K3 tanks; KE- SD2 - the values are presented as the mean±SD of the K4, K5, and K6 tanks; AE- SD1 - the values are presented as the mean±SD of the A1, A2, and A3 tanks; AE- SD2 - the values are presented as the mean±SD of the A4, A5, and A6 tanks;

Conductivity can be an effective way to estimate the fertilizer content via salts. In our experiment, electrical conductivity registered slightly higher values, with significant differences ($p<0.05$) between the fish-rearing units and plant units, but are in line with those reported by Al-Hafedh et al., 2008. Fish waste contains high levels of ammonia, which is toxic to fish but can be converted into nitrite and then nitrate by beneficial bacteria from the aquaponic system. Nitrate is a form of nitrogen that plants can readily absorb, and it serves as the primary source of nutrients for plants. In our experiment, it was observed that plants absorb nitrogen compounds. Choosing adequate plant density can help to reduce nitrogen levels in the water. Also, the good functionality of mechanical filtration and biological filtration is essential to maintain good water quality and reduce nitrogen compounds.

Plants Growth in Aquaponic System. Figures 2-5 and Table 2 present the plant's productivity.

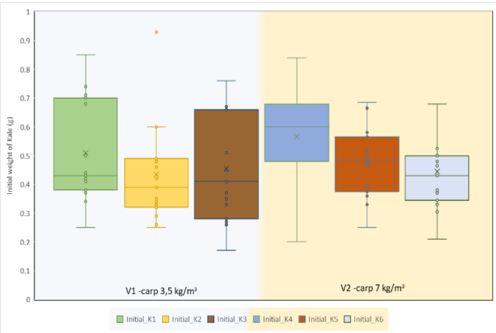

Figure 2. The distribution of the initial weight of kale

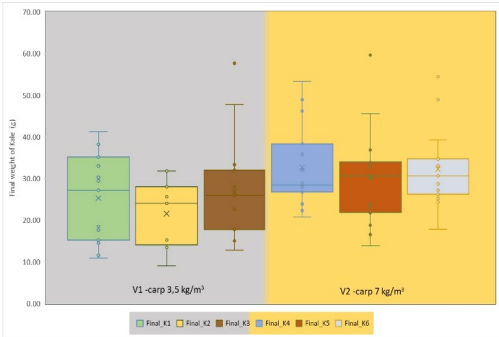

Figure 3. The distribution of the final weight of kale

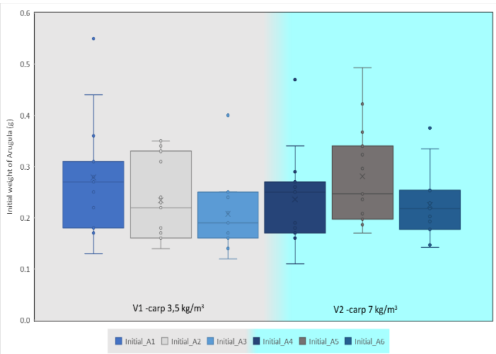

Figure 4. The distribution of the initial weight of arugula

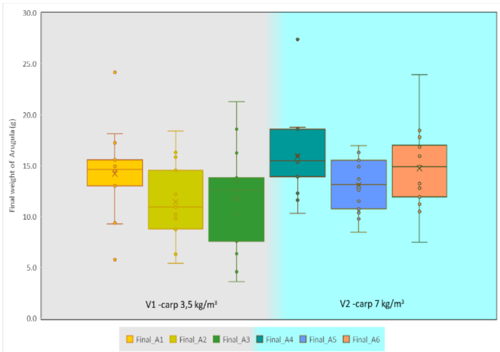

Figure 5. The distribution of the final weight of arugula

Table 2. Plant growth data from the aquaponic system

Parameter	Experimental moment	Kale		Arugula	
		SD1	SD2	SD1	SD2
Individual weight (g)	Initial	0.47±0.15	0.49±0.13	0.24±0.06	0.25±0.06
	Final	24.56±8.51	31.76±5.57	12.50±3.87	14.60±2.71
Plant biomass (g)	Initial	6.99±0.58	7.41±0.95	3.6±0.54	3.71±0.44
	Final	368.41±40.37	476.37±18.32	187.57±22.36	219.06±21.41
Root length (cm/plant)	Initial	2.16±0.05	3.08±0.09	2.04±0.03	2.15±0.04
	Final	13.5±3.69	19.16±3.71	15.45±3.6	18.53±2.66
The foliar surface of the leaf (cm^2/plant)	Initial	6.51±2.02	8.27±2.31	3.21±0.99	3.49±1.01
	Final	112.06±25.96	118.54±19.07	84.28±23.30	95.35±19.78

Note: *Kale*: SD1 - the values are presented as the mean±SD of the K1, K2, and K3 tanks; SD2 - the values are presented as the mean±SD of the K4, K5, and K6 tanks; *Arugula*: SD1 - the values are presented as the mean±SD of the A1, A2, and A3 tanks; SD2 - the values are presented as the mean±SD of the A4, A5, and A6 tanks.

The mean initial weight of kale was 0.47±0.15 g in SD1, respectively 0.49±0.13 g in SD2 ($p>0.05$), while arugula has lower individual weight: 0.24±0.06 g in SD1, respectively 0.25±0.06 g in SD2 ($p>0.05$). The mean final weight of kale was significantly higher ($p<0.05$) in the SD2 (31.76±5.57 g) in comparison with kale from the SD1 (24.56±8.51 g). Also, for arugula, a higher individual weight was obtained in the SD2 variant (14.6±2.71), but no statistical differences ($p>0.05$) were recorded (SD1-12.5±3.87 g). Lennard and Ward (2019), reported a final weight of arugula (after a growing period of 42 days in an NFT aquaponic system) of 10.7 g. Barbosa et al. (2020), reported for arugula a final weight of 14 g (plant density - 50 seedlings/m^2). In terms of the final plant roots, the statistical analysis revealed that the kale and arugula roots are significantly lower ($p<0.05$) in SD1 compared to those from SD2. The foliar surface of plants can be an indicator of the plant's ability to capture light and photosynthesize and offers information regarding the final yield (Pelil et al., 2018). The foliar surface of the leaves can vary depending on several factors, such as the density of the planting, and the growth conditions (Pérez et al., 2022). For kale and arugula, the result showed that the final foliar surface of leaves was higher in SD2, but there were no statistical differences ($p>0.05$) in comparison with SD1 (Figures 6-9). Kale has a final foliar surface of 118.54±19.07 cm^2/plant in SD2, respectively 112.06±25.96 cm^2/plant in SD1.

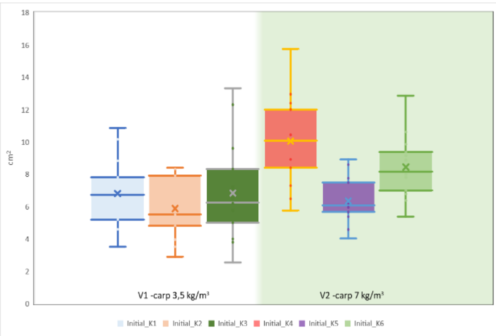

Figure 6. Leaf area of kale at the initial moment

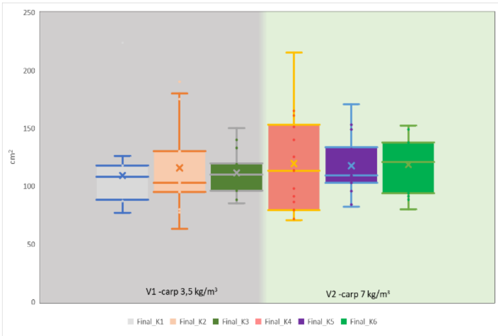

Figure 7. Leaf area of kale at the final moment

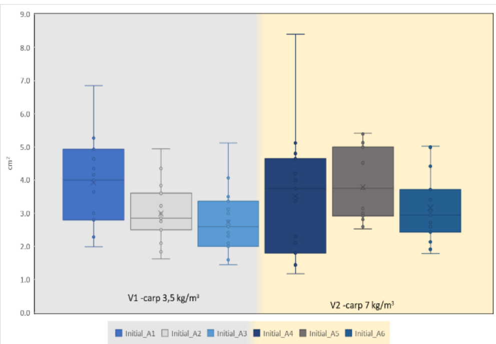

Figure 8. Leaf area of arugula at the initial moment

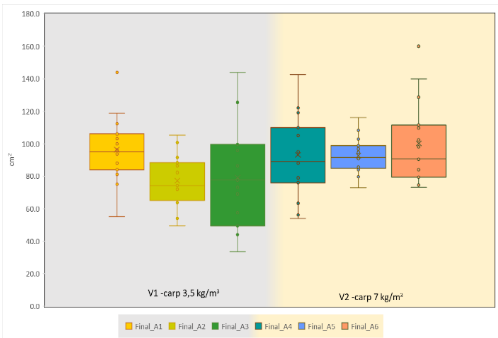

Figure 9. Leaf area of arugula at the final moment

Fish growth performance. Fish growth performance is presented in Table 2, and Figure 10. The initial mean weight of fish was 116.60±11.26 g at the SD1, respectively 116.63±14.52 g at the SD2. The results showed that aquaponics technology combined with different stocking densities of carp showed significant differences in fish growth ($p<0.05$). At the end of the experiment, the final fish weight was significantly higher ($p<0.05$) in fish stocked at a density of 3.5 kg×m^{-3}. The fish stocking density increased from the initial value

of 3.50 kg×m^{-3} to 5.72 kg×m^{-3}, respectively from 7 kg×m^{-3} to 9.18 kg×m^{-3}.
Also, regarding the main technological indicators (SGR, FCR, and PER) better values were obtained in carp stocked at lower density (3.5 kg×m^{-3}).

Table 2. Fish growth performance at the end of the experiment

Growth parameters	3.5 kg×m^{-3}	7 kg×m^{-3}
Initial biomass (g)	1749±15.42	3499±17.29
Initial biomass (kg/m^3)	3.50±0.41	7.00±0.54
The initial number of fish	15	30
Mean initial weight (g)	116.60±11.26	116.63±14.52
Final biomass (g)	2860±28.16	4700±34.26
The final number of fish	15	30
Mean final weight (g)	190.67±16.45	156.67±19.24
Biomass weight gain (g)	1111±15.65	1201±21.63
Final stocking density (kg/m^3)	5.72±0.26	9.18±0.63

Note: 3.5 kg×m^{-3} - the values are presented as the mean±SD of the B1, B2 and B3 tanks; 7 kg×m^{-3}- the values are presented as the mean values of the B4, B5 and B6 tanks.

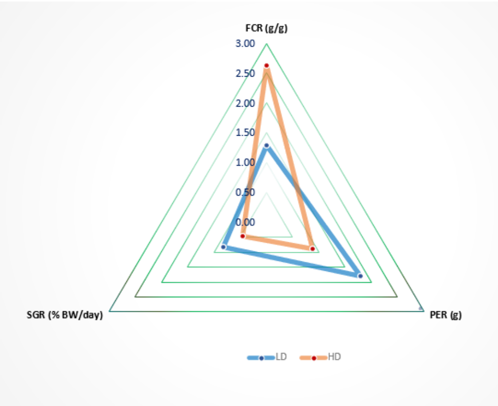

Figure 10. Specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) for common carp held in different stocking densities

The results of our study indicate that the fish were able to develop properly due to the adequate densities of fish and vegetables, which, overall ensured good water quality. In aquaponics, choosing the optimum stocking density is an important factor to optimize both fish and vegetable production without compromising the water quality. Overstocking of fish can lead to poor water quality (Di Marco et al., 2008) while the plants may not be able to consume all of the nutrients produced by the fish waste. On the other hand, if the fish stocking density is too low, there may not be enough waste to support optimal plant growth (Diver, 2006).

CONCLUSIONS

In the present study, the co-cultivation of common carp and two leafy vegetables (kale and arugula) was performed in an aquaponic system, for 41 days. Based on our results, it can be concluded that the larger biomass of kale and arugula obtained in the aquaponic system, indicates a big potential of these vegetables for higher production, in combination with common carp.
In our experiment, the studied plants act as a biological filter, suggesting that an aquaponics system is a potential method to grow vegetable biomass, being also a very eco-friendly aquaculture system.
Overall, the growth of common carp at a stocking density of 7 kg×m^{-3} in combination with kale and arugula (plant density of 51 plants×m^{-2}) showed better results in terms of plant growth, but the results were unsatisfactory in terms of fish growth.

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THE INFLUENCE OF THE EXPOSURE TIME TO THE PREVENTIVE TREATMENTS OF THE PIKE-PERCH (*SANDER LUCIOPERCA* L., 1758) EGGS, AGAINST FUNGAL INFECTION, DURING THE EMBRYONIC DEVELOPMENT PERIOD

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Abstract

Infection with Saprolegnia spp. is reported more and more frequently, becoming endemic in many aquaculture units, having a devastating impact on this sector, especially on embryonated eggs during the incubation period. This paper presents the way to prevent infection with Saprolegnia spp. by applying prophylactic treatments with formaldehyde to pike-perch eggs, during the embryonic development period. The experiments were carried out in triplicate, at SCDP Nucet, Romania, in 2022, at the artificial fish reproduction station. For prevention, formaldehyde solution was used, in a concentration of 1.7ml/l, the exposure time being different: in version V1 (control) of 10 minutes and in version V2 in which the exposure time was based on the respective water temperature 5, 10 and 15 minutes. The results were very good in the V2 variant with losses due to fungal infection of 4.8%, and good in the V1 variant (control) with losses of 14.6%.

Key words: *eggs, formaldehyde, fungal infection, pike- perch, Saprolegnia spp.*

INTRODUCTION

The development of aquaculture depends on the introduction of new species into the culture, as well as on the success of obtaining the fry necessary for stocking (Dobrota et al., 2022).

Compliance of disease and pest control treatment recommendations, especially in juvenile fish, ensures high production (Radu et al. 2022).

The pike-perch period of embryonic development in the hatchery takes place in April, when the water temperature stabilizes in the range of 12-14ºC. In the last decade, due to climate changes, during this period there are sudden changes in environmental parameters such as temperature, pH, organic substance etc.; they can induce outbreaks of saprolegnosis.

In fish culture, saprolegniosis is mainly a problem of eggs during the incubation period (Willoughby, 1970; Czeczuga & Kiziewicz, 1999; Hussein et al., 2001, Gieseker et al., 2006), even if sporadic outbreaks are reported in the situation where the environmental parameters register normal values, without sudden gaps (Thoen et al., 2011). In the past, the disease was adequately treated with malachite green, $Cu_2[(OH)_2|CO_3]$, an organic dye with biocidal effects. However, malachite green was banned for use in aquaculture when its use was found to pose a significant health risk to consumers due to its carcinogenic properties (Fitzpatrick et al., 1995; Kitancharoen et al., 1997).

Consequently, outbreaks of diseases with *Saprolegnia* spp., have increased significantly in the last decade in many areas of the world, with devastating impact on the aquaculture sector.

When incubating the eggs of pike-perch, losses due to microbial diseases can be significant and have significant financial implications. The mortality rate in embryonated eggs can reaches up to 80-100%. Treatments must be effective, safe and cost-effective (Radu et al., 2020).

MATERIALS AND METHODS

The research was carried out in 2022 in the Fish Culture Research and Development Station Nucet (SCDP Nucet), Dâmbovița County, Romania. The artificial breeding station is located in the main bed of the Ilfov brook, downstream of the Ilfoveni reservoir dam. The supply of technological water is carried out from

a settling pondlocated upstream of the reproduction station and its filtration is done through nytal fabric with a mesh of 0.1 mm.
The study material came from the natural-directed reproduction of the pike-perch, which was realized in earthen ponds, with the surface of approximate 1000 m^2. Spawning was done on breeding mats, made of fasciculate willow roots ("whiskers") (*Salix babylonica* L.).
The experimental variants were the following:
- Variant 1 (V1) - where the fertilized eggs were treated for 10 minutes once every 12 hours, was carried out in triplicate, in incubators I1, I2 and I3;
- Variant 2 (V2) - where the fertilized eggs were treated depending on the water temperature respectively for 5, 10 or 15 minutes once every 12 hours, was carried out in triplicate, in incubators I4, I5 and I6.

After harvesting the nests with fertilized eggs from the breeding ponds, they were carefully introduced into the nytal keeping net of the Nucet type incubators (Figures 1 and 2), where a permanent supply of water was ensured, at a flow rate of 8 l/min.

Figure 1. Detachment of nests with pike-perch fertilized eggs (Original photo)

Figure 2. Nest with fertilized eggs before being introduced into the Nucet type incubators (Original photo)

The capacity of the Nucet incubator is 140 litres, with surface water intake and bottom emptying, thus creating a vertical circular current of water, ensuring continuous fresh, well-oxygenated water for the eggs (Figures 3 and 4).

Figure 3. Nests with pike-perch fertilized eggs (Original photo)

Figure 4. The Nucet type incubators with nests of pike-perch fertilized eggs (Original photo)

The duration of the incubation was 7 days (depending on the water temperature), so the

incubation of the eggs started on 04/08/2022 and ended on 04/15/2022.
Considering the fact that the pike-perch is a very sensitive species in the first stages of development, the success of the reproduction expressed in the percentage of viability of the larvae depends to a large extent on the knowledge of the particularities of embryonic development in order to establish the appropriate technological interventions.
The application of prophylactic treatments to prevent infection with fungi was carried out by treating with a 37% formaldehyde solution in a concentration of 1.7 ml/l of water. The first treatment was administered 24 hours after fertilization in both experimental variants.
In the V1 variant, the duration of the treatment time was 10 minutes, without taking into account the water temperature.
In version V2, the duration of the treatment was dependent on the water temperature, as follows: 5 minutes at a water temperature of 8-10°C; 10 min at a water temperature of 11-13°C or 15 min at a water temperature of 14-16°C. The process was repeated every 12 hours, until the embryo surrounded the entire yolk sac, the tail reached the eyes, the pigmentation is accentuated, the movements of the embryo became more intense and the heart pulsations were observed.
For estimation of embryo survival and identification of fungi, during embryonic development, the collected samples were observed under a microscope (10X objective), on which occasion the viability and the degree of infestation were determined. Opaque eggs were determined to be dead, and clear eggs with adequate cell division were considered viable. Fungal infections are easy to spot, appearing as white or brown, cotton-like growths consisting of many small filaments.
The age or stage of embryonic development can be a significant factor in the management of the disease (Radu et al., 2020). In the early stages of embryonic development, stress can be more harmful and influence the survival rate. Understanding the development of *Saprolegnia* spp. is important for improving the hatch rate, which helps in the planning and duration of preventive treatments. During the embryonic development period, spawn samples were collected and used both to assess the hatch rate and to determine the number of hatched larvae.
It was found that, depending on the temperature of the water, after 10 to 14 hours after fertilization, the end of gastrulation takes place. In addition, the stage of development can be determined, during which the antifungal treatment continues. After 150-180 hours of incubation, at a temperature of 9-15°C, the process of embryonic development ended and hatching began, which lasted 18-24 hours.
Survival from spawn to larvae was determined with the formula (Olaniyi et al., 2013):

Sv (%) = Number of larvae × 100/Number of eggs for incubation

Losses due to infection with *Saprolegnia* sp. were determined as follows:

Losses (%) = Number of larvae × 100/Number of embryonated eggs

Under the conditions of the artificial breeding station at SCDP Nucet, the time required for the incubation of the eggs is directly correlated with the water temperature. Taking into account the exact moment of reproduction correlated with the evolution of temperature, the development of the embryos can be estimated. Temperature is an important environmental factor affecting eggs development, hatch rates and disease susceptibility. Throughout the incubation period, careful monitoring of the physico-chemical parameters of the environment was necessary. These measures can avoid the occurrence of mortality due to the accumulation of large amounts of organic matter, which is a food source for pathogens and can trigger diseases in incubation. Also, high levels of organic matter can reduce the effectiveness of formaldehyde.
The results of the experiment were used in the statistical analyses. The qualitative and quantitative data analysis was performed with MS Excel and represented by tables and graphs obtained from different types of results.

RESULTS AND DISCUSSIONS

Physico-chemical parameters of the technological water were monitored during the whole period of the experiments. The interpretation of the obtained results was carried out in accordance with the provisions of the "Regulation on the classification of surface water quality", correlated with the data from the specialized literature for waters used for fish farming (Ministry of the Environment and Water Management of Romania, Order no. 161, 2006) (Table 1).

Table 1. Average values of the physico-chemical indicators of water in the experimental period

Curt. No.	The chemical parameter		Unit of measure	Parameter values		
				Source	Incubators	Optimum according to quality standards
				The average of the year 2022		
1	pH		pH units	7.1	7.5	7-7.8
2	Alkalinity		mg/l	162	201	200-400
3	Calcium (Ca^{2+})		mg/l	46.8	44.6	90-120
4	Magnesium (Mg^{2+})		mg/l	19.8	21.4	10-40
5	Ca^{2+} / Mg^{2+}		mg/l	2.36	2.25	5
6	Organic substance		mg $KMnO_4$ /l	15	24.5	20-60
7	Oxygen		mg/l	10.6	8.4	5-12
8	Ammonia (NH_3^{+})		mg/l	lack	lack	lack
9	Nitrates (NO_3^{-})		mg/l	lack	0.24	2.5-4
10	Nitrites (NO_2^{-})		mg/l	0.001	0.002	0.03
11	Phosphates (PO_4^{3-})		mg/l	lack	0.05	0.05-1.5
12	Chloride	Cl^{-}	mg/l	8.65	8.36	30
		NaCl	mg/l	14.21	14.00	20
13	Ammonium (NH_4^{+})		mg/l	lack	0.018	0.5-1
14	Total hardness		(°D)	12.8	13.1	12

During the study period, the dissolved oxygen recorded variations that were between 8.4-10.6 mg/l. The pH of the water was between 7.1 and 7.5, the optimal range for the hatching of the eggs. The organic matter content of the water recorded values between 15-24.5 mg/l. The water temperature measured during the entire experiment recorded values in the range of 9-13°C (Figure 5).

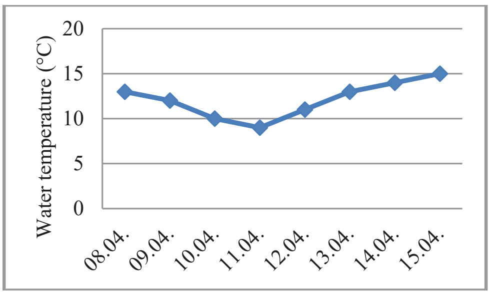

Figure 5. Evolution of the average water temperature

After harvesting the nests with fertilized eggs from the breeding ponds, they were very carefully introduced into the nytal keeping net of the Nucet type incubators (Figures 3 and 4), where a permanent supply of water was ensured, at a flow rate of 8 l/min.

From the specialized data and from the observations made, it was possible to find out the fact that the duration of the embryonic development process in the pike-perch eggs directly depends on the temperature of the technological water at which the embryonic development takes place. In Table 2 and Figure 5 it shows the duration of this process at the pike-perch depending on the water temperature. From the macro/microscopic observations it was found that, shortly after fertilization (1-2 hours), the diameter of the eggs varies between 1.2 and 1.4 mm. After 48-50 hours, under the conditions of embryonic development at water temperatures of 12-14°C, the embryo is in the gastrula stage. After 70-72 hours, the formation of the body of the embryo (in which 21 myomeres can be distinguished) and which half surrounds the yolk sac was observed. The eyes are slightly pigmented, the heart and blood circulation can be seen. After 4-5 days, a visible growth of the embryo became visible with the naked eye, whose body surrounds the yolk sac almost one and a half times, microscopically about 34 myomeres are visible. The eyes of the embryo are pigmented and have a brown colour. The primordia of the nasal orifice, the heart and the blood circulation can be distinguished, and inside the egg it is easy to observe the wriggling movements of the embryo, a sign that the moment of hatching is close.

Table 2. Average water temperature during the incubation period

Specification	Date								
	08.04	09.04	10.04	11.04	12.04	13.04	14.04	15.04	Total
Water temperature (°C)	13	12	10	9	11	13	14	15	-
No. baths/day	0	2	2	2	2	2	2	1	13
Exposure time (min.) V1	0	20	20	20	20	20	20	10	130
Exposure time (min.) V2	0	20	10	10	20	20	30	30	140
Amount of formaldehyde (liters) V 1	0	0.476	0.476	0.476	0.476	0.476	0.476	0.238	3.094
Concentration of formaldehyde (ml / water liter / treatment)	0	1.7	1.7	1.7	1.7	1.7	1.7	1.7	-

After the 7th to the 8th day, at the time of hatching, it was observed that the embryo surrounds the yolk sac twice, the eyes are strongly pigmented. Under the binocular microscope, small brown chromatophores were visible in the head area, on the yolk sac and to a lesser extent on the rest, while the digestive tube, the heart and pink blood and the fin fold were also visible. Figure 6 shows the larva of the pike-perch before the moment of hatching.

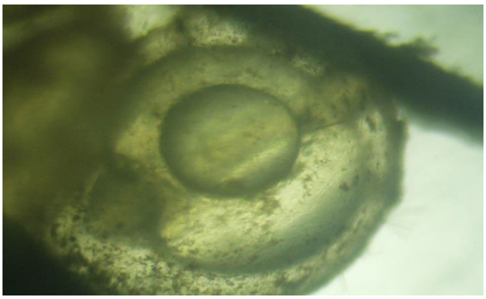

Figure 6. Pike-perch larva before the moment of hatching (Original photo)

During the incubation period, the average number of eggs deposited in the nests and introduced for incubation was calculated. The percentage of fertilization was determined by sampling the eggs deposited on the willow whiskers from each incubator and counting those with embryos and those not fertilized (which are opaque and whitish). Also, during this period, the percentage of hatching was determined, which was achieved in the phase when the heart pulsations and the movement of the embryo could be observed, and depending on the value of this percentage, the number of pike-perch larvae that were to hatch was determined.

The incubation period was 6-8 days at the average daily water temperature of 12-15°C.

After hatching, the remains of the willow whiskers were removed from the Nucet incubators, the larvae were kept in the incubators until they were 7-8 days old, during which time the water intake rate of the incubators was reduced to 4-5 l/min. Taking into account the fact that the larvae are sensitive and swim slowly, 2-3 frames of nytal were placed in the incubators for them to support and rest.

The comparative analysis took into account the values of the technological indicators from the experimental variant in which the treatments were carried out for 10 minutes/treatment, compared to the values of the technological indicators from the variant in which the treatments were carried out differently depending on the water temperature, respectively 5 minutes at the temperature of 8-10°C, 10 minutes at a temperature of 11-13°C and 15 minutes at a temperature of 14-16°C (Figure 7).

During the incubation period, the water temperature was recorded daily, the average number of eggs deposited in the nests and

introduced to the incubation, as well as the fertilization percentage, were evaluated.
During the incubation, the water temperature was recorded daily, the average number of eggs deposited in the nests and introduced to the incubation, as well as the percentage of fertilization was evaluated (Table 3 and Figure 8). The incubation period was 7-8 days at an average daily water temperature of 13.5°C.

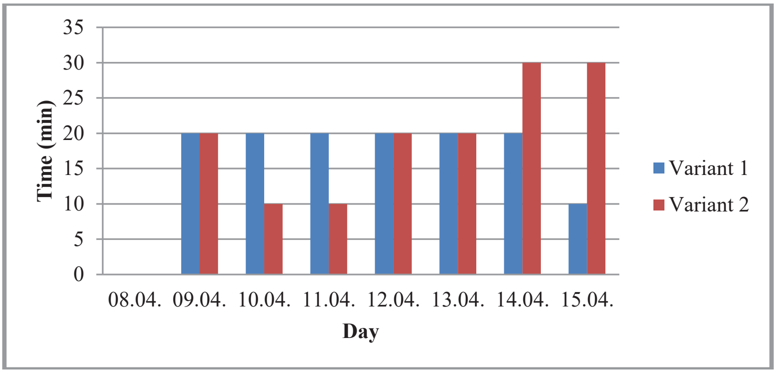

Figure 7. Evolution of treatment duration

Table 3. Biotechnological indicators obtained after treatmentof the embryonated eggs in the two experimental variants

Curt. No.	Biotechnological indicators	Unit of measure	V1	V2
1	Eggs for incubation	1000 pcs.	820.20	825
2	No. incubators	pcs.	3	3
3	No. eggs/incubator	1000 pcs.	273.40	275
4	Fertilization percentage	%	94.30 ± 3.77	94.20 ± 4.02
5	Number of fertilized eggs	1000 pcs.	773.4	777.2
6	Losses due to infection with *Saprolegnia spp.*	%	14.60	4.80
7	Hatching larvae	1000 pcs.	645.50	729.30
8	Incubation survival	%	78.70 ± 4.32	88.40 ± 4.27

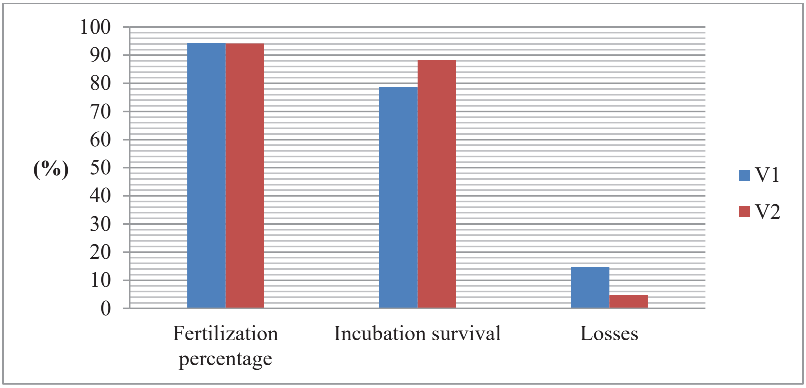

Figure 8. Fertilization percentage, incubation survival and losses recorded during the incubation period

The hatching rate was 88.4 ± 4.27% in the V2 variant and 78.7 ± 4.32% in the V1 variant.
The fertilization percentage had similar values in both experimental variants, with the following values: 94.3 ± 3.77% in variant V1 and 94.2 ± 4.02% in variant V2. Losses due to infection with *Saprolegnia* spp. recorded different

values, good results in the V2 variant (4.8%) and satisfactory in the V1 variant (14.6%).

CONCLUSIONS

The economic losses caused by fungal infestation can be severe and consequently disinfection measures must be taken. Each treatment has an economic value which includes the cost of treatment and the expected economic benefits.

Proper use of regulated products, some of which are quite expensive, can be important in preventing significant economic losses.

After the treatment carried out in the two experimental variants, the results demonstrated that the most effective treatment for preventing and combating *Saprolegnia* spp. infection is in variant 2 where the treatments were carried out depending on the water temperature.

Formaldehyde has the ability to inhibit the growth and spread of the fungus to live or dead eggs with superior results when taking into account the two essential factors in this process, namely: temperature and exposure time.

For the prevention and control of the disease, the treatments must be carried out rigorously, and the technological water must fit within the characteristics of the regulations in force from the point of view of environmental factors.

The correct use of regulated products, some of which are quite expensive, can be important in preventing significant economic losses.

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THE ROLE OF AMPHIBIANS IN MAINTAINING PARASITIC ZOONOSES (TREMATODOSIS) IN FISH IN THE REPUBLIC OF MOLDOVA

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Abstract

The paper presents data on the identification of the helminth fauna structure of ecaudata amphibians from Pelophylax and Bufo genera, and the determination of its role in maintaining parasitic zoonoses (trematodosis) in fish in the Republic of Moldova. As result of helminthological investigations 4 helminths species was established: Opisthioglyphe ranae Froelich, 1791; Tylodelphys excavata Rudolphi, 1803; Isthmiophora melis Shranch, 1788 and Neodiplostomum major Dubinina, 1950. This trematode species from a taxonomic point of view fall into a class (Trematoda), 3 orders (Plagiorchiida, Echisnostomida, Diplostomida), 3 families (Omphalometridae, Echinostomatidae, Diplostomidae) and 4 genera (Opisthioglyphe, Tylodelphys, Isthmiophora, Neodiplostomum). All this species of helminths comune in amphibians and fish, species of trematodes, for the fish are a negative impact, because causing various zoonosis. The need to write such a paper is due to the fact that the study of ichthyoparasites in the Republic of Moldova was carried for a long period of time (since 1963), but at the same time there was no discussion about the groups of organisms that contribute to the maintenance of the causative parasitic agents of various trematodes.

Key words: *amphibians, fish, Moldova, parasitic zoonosis.*

INTRODUCTION

Several causative agents of infectious diseases, including bacteria, viruses, parasites, and fungi, can be transmitted from animal to to another through different routes, including penetration through wounded or abrasive skin, ingestion, animal bites, vectors (insects), and animal-to-human contact (inhalation of respiratory particles or skin/mucous membrane contact) (Gauthier, 2015; Rahman et al., 2020). The pathogens that usually exist in animals can infect another animals, or humans either directly or via a vector (Wolfe et al., 2007).

Within aquatics, the general perception is that there are few zoonotic diseases considered as important (Shamsi, 2019). For those that are detected, the number of cases per year is small compared to other zoonotic diseases in animals or humans. While this might be correct, there is a possibility that this is an underestimate due to poor awareness and lack of monitoring and surveillance. However, for those that are diagnosed, the consequences can be severe, including death of host (Zorriehzahra & Talebi, 2021).

Emergence of zoonotic agents is a serious threat to global health and causes great damage worldwide (World Health Organization (WHO), 2021)

Amphibians can serve as a source of exposure to zoonotic agents for fishes. Although little published information is available regarding transmission of diseases or of parasitic agents from amphibians to fishes, several organisms have been identified as potential concerns, less amphibians. Awareness of the hazards of the formation and maintenance of a parasitic zoonosis in an ecosystem, require a sustainable helminthological management of all animal species, especially in amphibians, which can reduce sure the risk of infection fish from amphibians.

MATERIALS AND METHODS

Observation, collecting and obtaining data on the anurans from *Pelophylax* (*Pelophylax ridibundus* Pallas, 1771; *Pelophylax lessonae* Camerano, 1882; *Pelophylax esculentus* Linnaeus, 1758) and *Bufo* (*Bufo bufo* Linnaeus, 1758; *Bufo viridis* Laurenti, 1768) genera was performed in the area of the Republic of Moldova.

The helminthological analysis of biological samples was performed according to the standard method proposed by K.I. Skrjabin, which involves the examination of all the internal organs of the animal (Moravec & Skorıkova, 1998). Helminthological research of the parenchymal organs was performed with the help of compressors, and the digestive tract - by successive washes. The collection, fixing, determination and processing of the helminthological material was carried after the methods proposed by various authors (Koprivnikar et al., 2006; Koprivnikar & Poulin, 2009; Krone & Streich, 2000; May & Anderson, 1983; Moravec & Kaiser, 1994; Nickol, 1985; Okulewicz, 2008).

The determination of the helminthological material was performed according to standard methods (Ryzhikov et al., 1980).

In order to quantify the characteristic of helminthes contamination, the intensity indexes (II, specimens) was calculated - the minimum and maximum number of parasites of a species and the extent of invasion (EI, %) - the percentage of host contamination by a parasite species.

RESULTS AND DISCUSSIONS

Amphibians are one of the main components of the biocenosis, which directly contributes to the formation of parasitic zoonoses in fish. This important role attributed to amphibians in the formation and maintenance of various zoonotic agents specific to hydrobionts (fish) at least for a single developmental stage of helminths, egg, and larvae, adult is thanks to the fact that amphibians are a group of animals with an amphibiont way of life, thus populating both terrestrial and aquatic habitats. Due to this way of life of amphibians, for the entire annual life cycle (*Pelophylax* genus) or for part of the life cycle, the stage of lava development, until maturity, and the reproduction and laying of eggs every year must be in the aquatic environment (*Bufo* genus) are veridical transmitters of various groups of parasitic agents to various groups of hydrobionts, especially fish.

As established by Aho (1990), because amphibians have invaded a great variety of habitats and exhibit numerous patterns of life histories, reproductive modes, body size, and trophic relationships, they are excellent systems for exploring patterns and processes that influence the organization of helminth communities. By acting as generalist predators and as preys, amphibians play a role as intermediate and definitive hosts of a great variety of parasites in the aquatic and terrestrial food chains (Koprivnikar et al., 2012).

According to the helminthological investigations performed on amphibians from *Rana*, *Pelophylax* and *Bufo* genera, in area of the Republic of Moldova, the presence of 4 trematodes species specific to fishes was established: *Opisthioglyphe ranae* Froelich, 1791; *Tylodelphys excavata* Rudolphi, 1803; *Isthmiophora melis* Shranch, 1788 and *Neodiplostomum major* Dubinina, 1950. This trematode species from a taxonomic point of view fall into a class (Trematoda), 3 orders (Plagiorchiida, Echisnostomida, Diplostomida), 3 families (Omphalometridae, Echinostomatidae, Diplostomidae) and 4 genera (*Opisthioglyphe, Tylodelphys, Isthmiophora, Neodiplostomum*).

Tylodelphys excavata is a species, wich is characterized by the trixenic life cycle, with the obligatory participation of 3 hosts: 1 intermediate host, 2 intermediate host and the definitive host. Marita of *Tylodelphys excavata* parasitizes the intestines of storks of the genus *Ciconia*, which are also their obligate definitive hosts. *Planorbarius corneus* mollusk species, in the life cycle of this trematode, are first intermediate hosts, in whose body cavities the stage of cercar develops (Erhan & Gherasim, 2022; Szidat, 1935).

The metacercar of *Tylodelphys* sp. is a specific parasite freshwater fish (second intermediate host) and parasitizes in the vitreous body of the eyes.

On the territory of the Republic of Moldova, trematodes of the *Tylodephys* genus were detected in the following fish species: *Tinca tinca, Scardinius erythrophthalmus* and *Silurus glanis*. In these fish species, from 5 to 11 trematodes were recorded in one specimen.

According to the helminthological investigations carried out in amphibians, the intensity of invasion with this trematode species was established from 49 to 97 specimens in an individ (Table 1).

According to the helminthological research carried in fish, infestation was recorded in 27.3% of cases, and in amphibians, infestation was recorded in 52.8% of cases (Figures 1, 2).

Table 1. The intensity of invasion with trematodosis common to amphibians and fish

No.	Invazion	Amphibians	Fish	Amphibians	Fish	Amphibians	Fish
		Minimum		Maximum		Medium	
1.	*Tylodelphys* spp.	49	5	97	11	75	8
2.	*O. rane*	2	2	98	10	50	6
3.	*I. melis*	6	1	42	13	24	7
4.	*N. major*	1	6	6	57	3.5	31.5

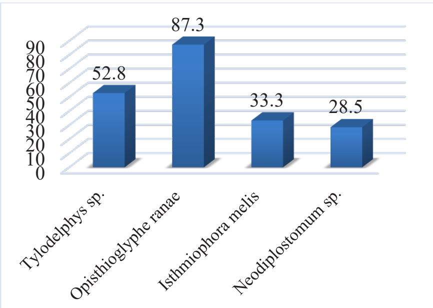

Figure 1. Parasitological indices of amphibians

Larvae of *Tylodelphys* Diesing, 1950 are major digenean pathogens of fish and amphibians. *Tylodelphys* spp. may induce mass mortality of fish and increase their susceptibility to predation. Even though *Tylodelphys* spp. cause substantial damage to aquaculture systems, surprisingly little is known regarding the taxonomy of this commercially important genus with a limited number of visible autapomorphic identification features.

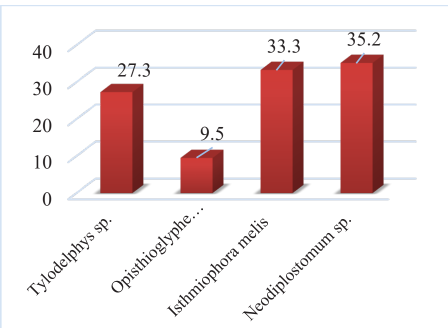

Figure 2. Parasitological indices of fish
(after Maritz, 1964; Moshu, 2014)

Opisthioglyphe ranae Fröhlich, 1791 trematode is widespread in amphibians. The first intermediate host are molluscs: *Lymnaea stagnalis, L. limosa, Galba palustris*, less often - *Radix ovata* and *R. auricularia*. Their infestation occurs by ingesting eggs containing miracidia. Sporocysts are formed in the snails, which produce cercariae. They are removed from the molluscs mainly in the morning hours. The second intermediate host is the larvae of amphibians, dragonflies, coleopterans and aquatic snails (mainly from the family Limnaeidae) in whose bodies, in the period of 6-10 days, metacercariae are formed.

Infestation of amphibians occurs through ingestion of molluscs, but also in cases of cannibalism. The infestation of fish with this trematode species is due to the fact that amphibians eliminate the eggs of this trematode in the aquatic environment - an environment strictly specific to fish, and the fish use intermediate hosts infected with this trematode species in their diet.

In our country, the trematode *Opisthioglyphe ranae* was determined by A. Moșu and Sokolov S. (2013) in the *Percuttus gleni* Dybowschi, 1877 fish species, in Cahul Lake and the affluents of the Prut River - the Draghiște River and the Racovaț River, where a prevalence of invasion was in 9.5% of cases (Figure 2).

In amphibians, the trematode species *Opisthioglyphe rane* is a species frequently encountered both in juveniles and in adults. When evaluating the obtained data, an intensity of invasion with this trematode species was established from 2 specimens in young amphibian forms to 98 specimens in an adult individual (Table 1), and the prevalence of invasion was recorded in 87.3% of cases (Figure 1).

Isthmiophora melis Schrank, 1788 is a species, wich is characterized too by the trigenic life cycle, with the obligatory participation of 3 hosts: 1 intermediate host, 2 intermediate host and the definitive host.

The first intermediate host is the freshwater mollusc *Lymnaea stagnalis*.

The second intermediate hosts are fish (*Carassius auratus*) and amphibians (*Pelophylax esculentus*).

The list of definitive hosts includes over 30 species of mammals including dogs, cats,

hedgehogs, otters, martens, rats, pigs, badgers, foxes and humans.
According to the helminthological data carried on fish on the territory of our country, it was found that fish are infected with *Isthmiophora melis* species in 33.3% of cases, and the number of specimens in an individual varies from 1 to 13 specimens (Maritz, 1963; Maritz, 1964; Moshu, 2014) (Table 1, Figure 2).
In the investigated amphibians, a prevalence of invasion was recorded in 33.3% of the cases, and the intensity of the invasion was recorded from 6 to 42 specimens in the specimen (Table 1, Figure 1).
Another trematodosis specific to both amphibians and fish is *Neodiplostomum major* Dubinina, 1950.
Neodiplostomum major species is a specific parasite of diurnal birds of prey. The intermediate hosts are the gastropod species *Planorbis planorbis* and *Planorbcirius comeus*.
Amphibians and fish serve as reservoir or paratenic hosts. Be that as it may, however, the fact remains that amphibians, as intermediate hosts, play a very clear role in the circulation of helminths and serve as a source of invasion of this group of fish parasites, but of the definitive hosts with this species of trematodes.
Moreover, the infection of amphibians occurs more frequently in their larval stage. The trematode has a wide range of reservoir hosts, which includes reptiles, birds and mammals.
This trematode parasitizes in the eyeball, often in the lens, less often in the vitreous body of fish. In fish, their infection can reach up to 35.2% of cases, and in one fish they can be found from 6 to 57 specimens (Moshu, 2014) (Table 1, Figure 2).
The losses caused by neodiplostomosis occur not only as a result of the death of fry, the deterioration of the body mass of sick fish and the quality of the meat, but also of their consumption by ichthyophage birds, which easily capture malnourished, cataract-affected fish (Mishanin, 2012; Novak, 2010).
In amphibians, this trematodosis was found only during the summer, with a prevalence of 28.5% of cases, and in one host can be found from 1 to 6 copies (Table 1, Figure 1).
These hazardous amphibians parasitic agents outbreaks reported (Table 1) indicate the importance of monitoring amphibians-derived zoonotic diseases.
All this species of helminths comune in amphibians and fish, species of trematodes (*Tylodelphys excavata, Opisthioglyphe rane, Isthmiophora melis, Neodiplostomum major*), for the fish are an negative impact, because causing various zoonosis.
Thanks to the determination of the presence of these species of trematodes in amphibians, their role in the formation and maintenance of outbreaks of fish parasitic zoonoses is demonstrated, and the massive losses caused by helminthosis are determined by their mass spread and affecting a large number of fish.
These losses can be expressed through the following causes: the mass death of fish, especially the fry; in many helminthiasis the fish is cachexic, remains undeveloped, stagnating the process of obtaining fish production.
Fish acquired these parasites while feeding on benthic invertebrates (mollusks, aquatic insect larvae, leeches) and free-swimming cercariae.
Therefore, as can be concluded from the above data, the circulation of helminths from a structural and functional point of view is a complex dynamic process, based especially on intermediate hosts (cercari host, metacercari host). With their participation, additional channels are formed, through which a large part or even the entire flow of invasive larvae from intermediate hosts and unique duplicate systems is created (Sharpilo, 1979). Because of them, a number of helminth species could apparently survive significant historical periods and reach our time from other geological epochs, despite the elimination of the primary definitive hosts by natural selection.
The high degree of infestation with these trematodosis specific to fish, demonstrates the role of amphibians as eliminators of the invasive stages of helminths with a dangerous impact both for fish and for human health. The probability of reducing the number of amphibians certainly extends to the increase the degree of infestation with various trematodosis in fish, but other animal species (eggs, larvae, adults).

CONCLUSIONS

It has been studied the helminth fauna of amphibians in the *Pelophylax* and *Bufo* genera and the determination of its role in maintaining parasitic zoonoses (trematodosis) in fish in the Republic of Moldova.

As result of helminthological investigations 4 helminths species was established: *Opisthioglyphe ranae* Froelich, 1791; *Tylodelphys excavata* Rudolphi, 1803; *Isthmiophora melis* Shranch, 1788 and *Neodiplostomum major* Dubinina, 1950. This trematode species from a taxonomic point of view fall into a class (Trematoda), 3 orders (Plagiorchiida, Echisnostomida, Diplostomida), 3 families (Omphalometridae, Echinostomatidae, Diplostomidae) and 4 genera (*Opisthioglyphe, Tylodelphys, Isthmiophora, Neodiplostomum*).

All this species of helminths comune in amphibians and fish, species of trematodes, for the fish are a negative impact, because causing various zoonosis.

In this sense, the role of amphibians in the formation and maintenance of outbreaks of fish parasitic zoonoses was demonstrated, and the massive losses caused by helminthosis are determined by their mass spread and affecting a large number of fish species.

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NUMBERS AND POPULATION DYNAMICS OF THE WHITE STORK (*Ciconia ciconia*) COLONY IN BELOZEM - THE EUROPEAN WHITE STORK VILLAGE IN BULGARIA - IN 2020-2022

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Abstract

The White Stork is one of the iconic bird species that is easily recognized by humans as it inhabits and nests in settlements as well as other places and structures located near lakes, rivers, dams, rice fields, wet meadows, and others. Given its feeding habitats, the species is a typical farmland bird. In many settlements in Bulgaria, including the village of Belozem, several dozen nesting pairs have been established, and this number is significantly higher than the average for the country. Some of the largest rice fields in the country, which are key foraging habitats for the species, are located around Belozem. In the village of Belozem, the stork colony formed on the roof of the local school is very impressive and counts over 20 nests, which represent almost half of the pairs nesting in the village - about 40 in total. In the current study, 41 to 53 stork nests were recorded, and 35-37 pairs successfully reared at least one juvenile each. The number of fledglings leaving the nest ranged from 94 to 115.

Key words: *breeding parameters, farmland birds, rice fields.*

INTRODUCTION

The White stork (*Ciconia ciconia* Linnaeus, 1758) is one of the iconic bird species that is easily recognized by humans as it inhabits and nests in lowland settlements without the central parts of large cities (Simeonov et al., 1990), places, and structures located near lakes, rivers, dams, rice fields, mesophytic grasslands, wet meadows, abandoned arable fields, etc. (Milchev et al., 2013; Petrov et al., 2015). Considering its foraging habitats, which it uses, the species can be described as a representative of farmland birds (Tobolka et al., 2012). The species is a top predator in these habitats, feeding on voles, fish, amphibians, reptiles, etc., thus storks indicate the abundance and diversity of other species in agricultural lands. This defines it as particularly sensitive to changes in agricultural areas, where with the intensification of agroecological practices in the last century, the capacity of ecosystem services from these sources has significantly decreased (Emmerson et al., 2016). To reduce the negative impact of these factors, compensatory measures could be applied, such as the maintenance of territories with low pesticide treatment, the construction of artificial reservoirs and flooded areas, the securing of risky nests, and the installation of nesting platforms. Along with conducting regular monitoring, the direct environmental protection measures described above are extremely important for the conservation of the species, especially in areas with aggregation of breeding pairs.

In many settlements in Bulgaria, including the village of Belozem, several dozen nesting pairs have been established, and this number is significantly higher than the average for the country. In Belozem, Rakovski municipality, are located some of the largest rice fields in the country, which represent key foraging habitats for the species. The White stork colony in Belozem formed on the roof of the local elementary school "Geo Milev" counts over 20 nests, which represent almost half of the pairs nesting in the village - about 40 in total. The colony on the roof (more than 20 nests) is the largest gathering of stork nests in the country, in a single location with total area of about 1200 m^2. In this regard, after proposal by Green Balkans NGO since 2005, the village has been included in the European Stork Villages Network, uniting settlements from different countries, where not only the number of these birds is high, but also the local communities take care of their conservation, and maintain their habitats (www.storkvillages.net). The "European Stork Villages" have been designated for their exemplary dedication to the protection of the White stork. Since 1994 the EuroNatur foundation has honoured 15 villages in 15 European countries for their engagement in White stork and nature conservation with the title "European Stork Village" (Figure 1). With that initiative EuroNatur has set up a movement to help counterbalance the habitat loss for storks in Europe.

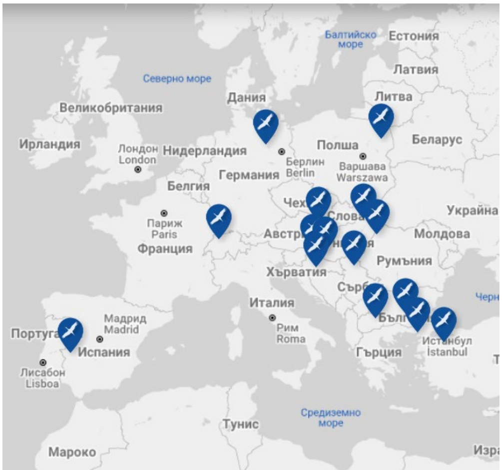

Figure 1. Map of European Stork Villages.
Source: www.storkvillages.net

Implementing monitoring of this species using standard field survey methods is greatly facilitated due to the proximity of its breeding habitats to human settlements. The national number of the White Stork in Bulgaria, based on national censuses of White Stork in Bulgaria during 1994-2015 varies as follows: 1994-1995 - 4228 breeding pairs; 2004-2005 - 4818 breeding pairs; 2014-2015 - 5825 breeding pairs (Petrov 1997; Petrov et al., 2007; Cheshmedzhiev et al., 2016). In some settlements in Bulgaria - villages Belozem, Dragushinovo, Kulata, Belchin, Samuilovo – several tens of pairs are nesting, and thus the abundance of White storks there is significantly higher than the national average (Cheshmedzhiev et al., 2016). According to the Methodology for national counts of the White stork, in most cases, the assessment is based on a single visit to the nests, as well as conducting a poll survey among local people.

In Bulgaria, surveys on nesting behavior and breeding parameters are presented from several successive national censuses (carried out once every ten years) of the number and nesting distribution of the species. The earliest data at the national level are from the second half of the last century - Milchev & Stoyanova (1986) and Michev et al. (1989), when Bulgaria joined the international initiative of White stork census. Afterwards, national censuses of the species were conducted once every 10 years: 1994-1995 (Petrov, 1997); 2004 - coverage of about 1/3 of the country's territory (Kmetova & Michev, 2006); 2004-2005 (Petrov et al., 2007); and 2014-2015 (Cheshmedzhiev et al., 2016).

MATERIALS AND METHODS

The object of the study is the White stork (*Ciconia ciconia* Linnaeus, 1758). It belongs to the Order Ciconiiformes, Family Ciconiidae. The species is breeding and migratory, transiting, and sometimes wintering on the territory of Bulgaria. In the Red Data Book of Bulgaria, the conservation status of the species in Bulgaria is determined as Vulnerable (VU) (Petrov et al., 2015). According to national legislation, the species is protected and listed in Biodiversity Act's annexes 2 and 3.

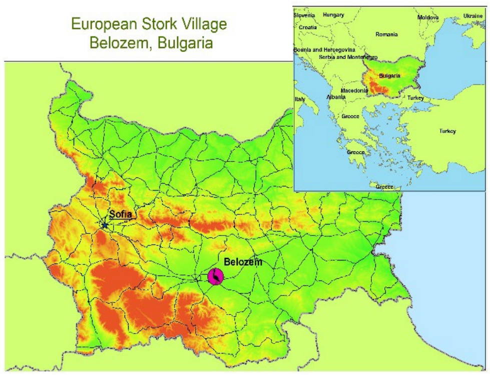

Figure 2. Location of Belozem

The study was conducted in 2020-2022 in the village of Belozem (Figure 2). It is situated in the central part of southern Bulgaria, in the Thracian lowlands close to the largest Bulgarian river - Maritsa. This area is known in the country for its rice production and its wet soils. Each year more than 400 ha of rice fields are cultivated here. The land of the Belozem village, which represents the main feeding grounds of the village's stork colony, covers an area of 4200 ha. The region includes also small parts of NATURA 2000 sites in Bulgaria: SPA "Maritsa - Parvomay" (BG0002081, Area: 11,512.83 ha) and SCI "Reka Maritsa" (BG0000578, Area: 14,696.07 ha) (MOEW, 2015) (Figure 3).

Figure 3. Rice fields in Belozem area

For the present study, detailed monitoring of all nests in Belozem was carried out, with a minimum of 8 visits during each of the three breeding seasons in the period 2020-2022. The field surveys and observations of the White stork nests are carried out according to standard methodology, reporting absolute values from each point observation. The data were collected in a standard field form as well as a specialized mobile application - Smart StorkBelozem, part of SmartBirds. Implementation standard established methods are used, including observations and follow-up with binoculars (Zeiss Conquest HD 8 x 42), field scope (Swarowski 80HD), camera (Nikon D 71000, Nikon AF-S Nikkor 200-500 mm), Drone MAVIC PRO with camera Sensor 1/2.3 (CMOS) (Figure 4), and video surveillance which is constructed on a similar type to that described by Stamova et al. (2017), which allows monitoring of several nests simultaneously without disturbing the birds in the nests (Fig. 5).

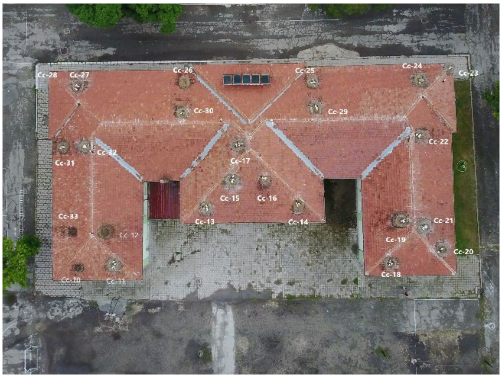

Figure 4. Location of the nests on the roof of the school in Belozem village photographed by a drone

The reporting of breeding parameters was carried out using the following indicators: **HPa** - stork pair present during the breeding season at the nest; **HPm** - stork pair with fledged offsprings; **HPo** - stork pair without fledged offsprings; **JZG** - total number of fledged offsprings in the area; **JZa** - the average number of fledged offsprings per breeding pair HPa; **JZm** - the average number of fledged offsprings per HPm (Wobus, 1963).

Figure 5. Video surveillance of the White stork nests in Belozem village

The assessment of this type of indicator has also been used in several other studies on the subject, both at the national (Petrov 1997; Kmetova & Michev, 2006; Petrov et al., 2007; Cheshmedzhiev et al., 2016) and international level (Nowakowski, 2003; Daniluk et al., 2006; Denac, 2010; Kósa, 2015). Back in the middle of the last century, in Germany, a study of the same breeding parameters in the period 1956 – 1960 was carried out (Wobus, 1963). This is a

good basis for comparability and analysis of the data obtained.
The raw data were processed and then analyzed by ANOVA statistical models.

RESULTS AND DISCUSSIONS

During the three years of the current study (2020-2022), 41 to 53 stork nests were recorded (HPa), and 35-37 pairs successfully reared at least one juvenile each (HPm) (Table 1, Figure 6).

Table 1. Breeding parameters of the White stork colony in Belozem village during the study period

Parameters/Year	2020	2021	2022
HPa	41	47	53
HPm	37	35	36
HPo	4	12	17
JZG	115	94	111
JZm	3.108	2.686	3.083
JZa	2.805	2	2.094

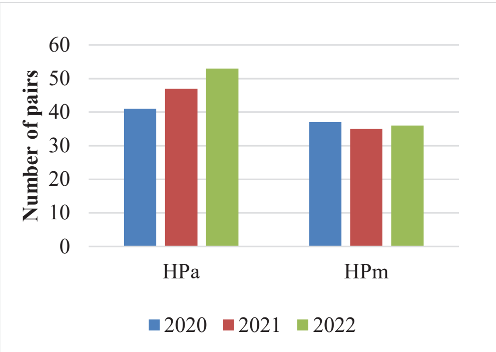

Figure 6. The number of pairs (HPa) and pairs with fledged offsprings (HPm) of the White stork *Ciconia ciconia* in 2020-2022 in Belozem village

Despite the increase in the number of occupied nests (HPa) by 12 in only three years, the number of pairs with juveniles rearing success (HPm) keeps approximately close values of 35-37 pairs per year. There is a clear trend in the number of pairs with no reared juveniles (HPo) concerning occupied nesting territories. As the number of occupied nests increases, the number of pairs with no reared juvenile also increases, from 4 to 17 (Table 1, Figure 7):

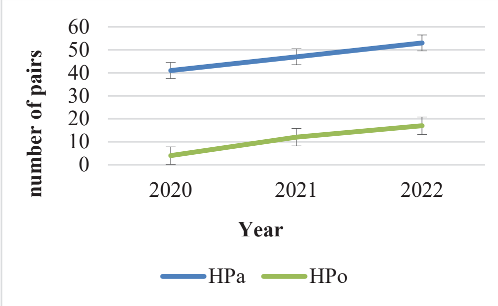

Figure 7. The trend of breeding parameters HPa and HPo for the period 2020-2022

On average, from each occupied, successful nest (HPm, N = 108), 2.9 ± 0.23 nestlings were fledged (JZm N = 320) during 2020-2022. In most nests (67%), 3 fledglings were found (Figure 8). Our findings are in unison with the results obtained from Daniluk et al. (2006) in Poland.

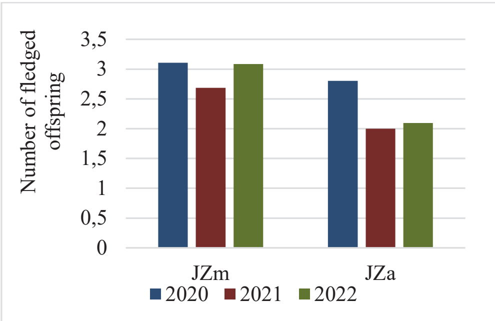

Figure 8. Comparison of fledged offsprings regarding pairs for the period 2020-2022

There is a clear tendency in increasing the numbers in our country, as well as in other European countries such as France, Netherlands, Germany, and Italy (Chodkiewicz & Sikora, 2020). Being one of the 20 stork species in the world, the White stork is the most studied one - the studies on the White stork are more than one-third (37.5%) of all known publications on the storks (family Ciconiidae) (Gula et al., 2023).
Table 2 and Figure 8 present the average values of the breeding parameters JZa and JZm in Bulgaria, based on data from national censuses of the species during 1994-2015, and the average values for each of the parameters JZa and JZm in the present study during 2020-2022

(current study Table 2). In total, the data covers 25 years.

Table 2. Breeding parameters JZa and JZm in Bulgaria 1994-2022

Source	Current study	Cheshmedzhiev et al., 2016	Petrov et al., 2007	Kmetova & Michev 2006	Petrov 1997
JZm	2.96	2.73	2.8	2.75	2.8
JZa	2.3	2.44	2.5	2.48	2.5

The average values for each of the presented periods are close and comparable, which confirms the reliability of the data in the period studied by us. There are no significant differences between seasons in the average number of fledglings per successful breeding pair ($F_{17,8402}$=19, $P > 0.005$). The best season was in 2020 when on average 3.1 nestlings fledged, and the worst was in 2021, with 2.68 fledglings per successful breeding pair (JZm) (Figure 8).

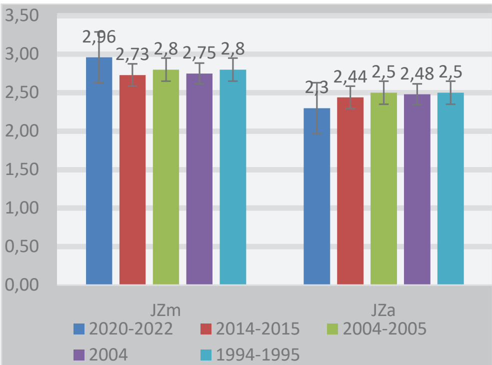

Figure 9. Average values for breeding parameters JZa and JZm for the period 1994-2022

The number of fledglings does not depend entirely on the number of successfully nested pairs (HPm). Most likely, environmental factors also have influences such as temperature, food availability, etc.

Average values for breeding parameters JZa and JZm in the colony of Belozem are relative to national data collected from the last three national censuses of White stork in Bulgaria during 1994-2015. Still, the average values of JZm for the three years in Belozem are slightly higher than, the same parameter for any other of the studied periods at the national level.

Statistically significant differences were observed between years 1994-2022 (Figure 9) at national level in average number of fledged offspring per successful nesting pairs and average number of fledged offsprings per stork pair present during breeding season at the nest ($F_{5,3176} = 43{,}5601$, $P < 0.0001$).

CONCLUSIONS

Many factors are crucial for breeding success and vary between seasons and stork pairs.

For the farmland birds, food abundance, lack of disturbance, and ecologically friendly agricultural practices are essential. Fluctuation in breeding success depends also on environmental conditions and varied between years.

The large aggregation of a high number of birds at the same time in the same place, in the Belozem village, determines the high importance of this area for the conservation and survival of the birds of this species, and at the same time shows the high sensitivity of the individuals to changes in the quality of their habitats. This also necessitates the implementation of direct environmental protection measures targeting protecting the nesting substrate, reducing disturbance, and sustainable management of habitats.

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We wish to express our gratitude and pay tribute to our late colleague Doncho Kirov for the dedicated efforts and demonstrated skills in the work for study and conservation of the White stork in Belozem - the Bulgarian representative in European Stork Villages Network.

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ADAPTIVE RESPONSE OF CARP TO AQUATIC ENVIRONMENT CHANGES: A CASE STUDY

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Abstract

As part of a larger study, the present work highlights the way fish specimens affect the parameters of the aquatic environment, which is essential in fish farming. Six common carp (Cyprinus carpio) were subjected to an experiment where they were transferred to new aquatic environments, while two specimens remained in the water of origin. Each individual modified the initial values of the aquatic environments by balancing them to create a framework conducive to their survival. During the experiment, no feeding methods were applied, and the individuals' intervention on the water was strictly observed. The results demonstrated the high degree of adaptability of the species, but there was also an early case of fatality, which was justified. The intervention of the individuals was highlighted by the value of the parameter PO_4*, which exceeded the recommended value in aquaculture. However, the value was identical when each individual was removed from the experimental module.*

Key words*: aquatic environment, Cyprinus carpio, freshwater aquaculture, transfer.*

INTRODUCTION

Worldwide, increased attention is being paid to genetic studies of aquatic environments. Environmental DNA (eDNA) represents the trace that individual organisms leave in the environment they inhabit (Wilcox et al., 2013).
Most genetic studies on aquatic macroorganisms have been based on water sample DNA (Minamoto et al., 2012; Miya et al., 2015; Ushio et al., 2017; Goldberg et al., 2018; Nelson-Chorney et al., 2019; Ishige et al., 2021), but other studies have focused their methods on underwater sediment DNA (Turner et al., 2015; Shaw et al., 2016; Valentini et al., 2016; Buxton et al., 2018). To date, a comparison of core properties between sample types has only been performed for fish DNA (Turner et al., 2015). Until now, the choice of applying one of the two methods (DNA extracted from the water sample or from the sediment) was aimed at accuracy and information only for fish DNA (Sakata et al., 2020) and only from environmental samples from seawater (Sutcliffe & Sharp, 1968; Maeda & Taga, 1973).
In the 1980s, specialists began sequencing DNA extracted from water and soil samples (e.g., Torsvik, 1980; Ogram et al., 1987; Bailiff & Karl, 1991). Microbial strains could not be cultivated in the laboratory, and researchers became interested in developing advanced techniques and technologies for their study (Henne et al., 2000; Michotey et al., 2013).
The intervention of fish in the aquatic environment is reflected in genetic material found in sediments and their composition (Wilcox et al., 2013), just as water parameters affect the genetic structure of the individuals that inhabit it (Markov et al., 2021). After describing the issue of adaptability, it is important to note that individuals are capable of adapting over time, typically through several generations (Witt & Huerta-Sanchez, 2019). However, problems arise when fish are manipulated and the environment of origin is changed in order to complete the technological process pursued within the economic flow.
In fish farms, water is considered the living environment of fish populations (Pilakouta et al., 2022), and it becomes an important and determining factor in the quality of fish products intended for human consumption. At the same time, for the economic yield of the farms, the

quality-to-price ratio increases when there are no losses.

Significant losses occur during the transport of biological material from one farm to another without considering the parameters of the aquatic environments, both those of origin and those of transfer. In the case of reproduction, even minor differences between the aquatic environment of the fry and the aquatic environment in which it is transferred can result in losses of up to 80% of the number of specimens transferred, spontaneously or over time (Lostun et al., 2002).

MATERIALS AND METHODS

Materials

The aim of this study was to demonstrate the influence of individual fish on their aquatic environment, with a particular focus on the common carp (*Cyprinus carpio*).

The biological material used in the experiment consisted of eight *C. carpio* specimens obtained from three different farms, specifically selected based on the distance between them and the resulting differences in the chemical and biological aspects of their aquatic environments.

The first farm, from which three scale-bearing *C. carpio* specimens were obtained, was the Moldavia Delta Complex (Larga Jijia) located at 47°21'14.7"N 27°22'12.4"E. This complex has been designated as a protected area of community interest by the Order of the Minister of Environment and Sustainable Development No. 1964/2007 on the establishment of the protected natural area regime of sites of community importance (Official Gazette No. 98, 2008). The complex consists of several lakes covering approximately 1250 hectares, where the common carp, Prussian carp, silver carp, and bighead carp are prevalent. As a polyculture farm, other species such as sheatfish, perch, and pike can also be found. The main activity of the farm is freshwater aquaculture, which includes not only fish growth but also reproduction. The farm also features a patented, scaleless variety of *C. carpio* known as topless Movileni carp.

The second farm from which 3 specimens of *C. carpio*, a variety with scales, were brought to the laboratory, was the Acvares Fish Farm at 47°19'23.8"N 27°32'06.4"E. It has an area of 237 hectares, of which 170 hectares is productive area. The farm produces fish from the species carp, including *Cyprinus carpio*, *Ctenopharyngodon idella*, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*, *Silurus glanis*, *Sander lucioperca*, and *Polyodon spathula*, but the main species is common carp. The farm produces one summer fry (3 months old), two summer fry (15 months old), fish for consumption (1.5-2 kg), and selected breeder lines. Production and reproduction are carried out in ecological conditions and with the best quality feed, without the use of chemical fertilizers or manure.

The third farm from which 2 specimens of *C. carpio,* the scaled variety, were harvested for the study was Bârca Fish Farm at 47°04'40.1"N 27°30'05.7"E. It has an area of approximately 70 hectares of water divided into 2 ponds of 18 hectares at 47°04'39.1"N 27°30'04.1"E and 23 hectares at 47°04'40.9"N 27°29'12.6"E, respectively, and 6 ponds for fry. The main activity of the farm is freshwater aquaculture, but it does not deal with the reproduction of fish material. The natural setting is preserved, and the feeding of the fry is carried out by the natural food created in the ponds during the cold seasons. The ponds are emptied and cleaned in the fall, after harvesting the saplings. Natural fertilizer is applied, represented by manure, and in the spring, the areas intended for the fish fry are flooded with water from the major accumulation.

The distance between the farms, as well as the technologies applied within each one, assured us that the fish material used in the study was healthy and resistant. The selection of individuals was carried out at the time of harvesting from each farm, following their transport and transfer under minimal stress conditions.

The experiment was carried out for 16 days, calculated from the release of the specimens in the studied environments until the last specimen showed signs of lethargy. The specimens of common carp (*Cyprinus carpio*) in the study were of the scaly variety, aged 18 months (one year and one summer), and presented the metric qualities detailed in Table 1.

Table 1. Performance of the analysed characters in the specimens used in the study

Farm	Exemplary	Body mass (kg)	Total body length (cm)
A-The Moldavia Delta	A_1	2.00	36
	A_2	1.98	35
	A_3	2.00	36
B-Acvares	B_1	2.10	37
	B_2	2.00	36
	B_3	2.10	37
C-Piscicola Bârca	C_1	2.10	35
	C_2	2.30	36

Methods

Samples were collected from each aquatic environment to establish the initial parameters of the water from which the specimens used in the experiment were collected (Table 2). The water was collected in a transparent glass container with a capacity of 1 liter, and the analyses were carried out at each farm during the collection of the biological material before its selection. To determine the parameters of interest, 1000 ml of water were collected and analysed from each aquatic environment. The parameters of interest included KH, GH, pH, NH_4, NO_2, NO_3, PO_4, SiO_2, Fe, Cu, and O_2, and their values were determined using a freshwater test laboratory called "JBL - Pro Aqua Test Lab".

After analysing the original aquatic environments, the individuals were distributed into different aquatic environments, some of which were new for the specimens analysed (Table 2).

Table 2. Distribution of specimens in aquatic environments within the laboratory

Fish	Aquatic environment (100 l water)
A_1	M^A
B_2	
C_1	
A_2	M^B
B_1	
C_2	
A_3	M^P
B_3	

The experiment was conducted at a constant temperature of 6°C in the aquatic environments, and each individual was released into 100 liters of water. Since the specimens were at a constant temperature of 6°C, they had no appetite (Official Gazette, Romania, 2008). In order to avoid any disturbances to the aquatic environment caused by external factors, including food, the influence of individuals on the environment was strictly monitored.

For aeration in each aquarium, Stream 480 submersible pumps with adjustable air flow were used, with the aim of minimizing the intervention on the aquatic environments. The pumps had a power of 4.3 W and a capacity of 520 L/h, which met the requirement for aeration in 100 L of water in each aquarium.

The data were statistically analysed, and the Tukey Test was used to determine the significance of the differences between the samples, whenever necessary, based on the results of the Fisher Test. The Tukey Test is a statistical test used for multiple comparisons between means (Tukey, 1949; Kramer, 1956). It is used to determine whether there is a significant difference between two or more means. The test is based on the Studentized Range Distribution and is also known as the Tukey-Kramer method or the Tukey HSD (Honestly Significant Difference) test.

The Tukey test is conducted after performing an analysis of variance (ANOVA) and if the F-test is significant. The formula for the Tukey test statistic is: q = (Yi - Yj) / SE, where q is the Tukey test statistic; Yi and Yj are the means being compared; and SE is the standard error, which is calculated as: SE = sqrt (MSW / n), where MSW is the mean square error from the ANOVA, and n is the number of observations.

The critical values for the Tukey test depend on the number of means being compared and the number of degrees of freedom. These critical values can be found in a table of the Studentized Range Distribution.

The Fisher Test, also known as Fisher's Exact Test, is a statistical test used to determine the significance of the association between two categorical variables (Fisher, 1922). It is used when the sample size is small, and the expected values are less than 5. The test is based on the hypergeometric distribution.

The formula for the Fisher test statistic is:

p = (r! * (n1 - r)! * (n2 - k)! * (N - n1 - n2 + k)!) / (n1! * n2! * (N + 1)!), where:

p is the p-value;

r is the number of successes in sample 1;

n1 is the sample size of sample 1;

k is the number of successes in sample 2;
n2 is the sample size of sample 2;
N is the total sample size.

The critical value for the Fisher test can be found in a table of the Fisher's Exact Test distribution.

RESULTS AND DISCUSSIONS

Results

The final parameters of the aquatic environments that were studied are listed in Table 3. The ", which represents the final values of the water parameters of the aquatic environment from A farm, were determined by A_1, B_2, and C_1 fish that were released into this water during the study. The " is represented by A_2, B_1, and C_2 fish that were released into B farm water at the end of the experiment. The next columns represent ", which shows the final values of the bottled still water that was populated by A_3 and B_3 fish, and the last column is for the aquatic environment of C_1 and C_2 carps, the water they originated from.

In the first case, the specimen that was released into the M^A water did not change the environment but was only retained in laboratory (aquarium) conditions. The change in parameters (Figure 1) was interpreted as a response to stress stimuli, with the individual being classified as wild. The reduction of the movement surface to 100 l of water represented a strong stress factor.

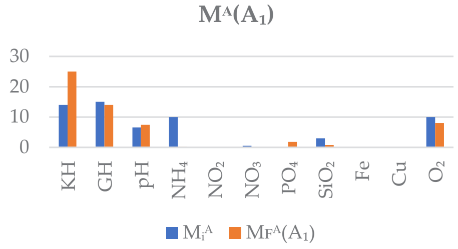

Figure 1. Average values of M^A parameters with fish A_1

The consumption of O_2 shows the effort made by the specimen in adapting to the new conditions, resulting in changes to the KH and pH levels, without exceeding the recommended limits in aquaculture. The SiO_2 content decreases, falling within the maximum recommended limit of up to 1.2 mg/l, while PO_4 increases. Phosphorus, being the structural link in genetic material (DNA and RNA) and the component element of phospholipids in cell wall membranes, as well as in the structure of scales (Bud et al., 2010), marks the individual's intervention on the environment.

Table 3. Average values of the studied aquatic environments parameters

Aquatic environment		Fish				Fish				Fish		Mc
Unit		A_1	B_2	C_1		A_2	B_1	C_2		A_3	B_3	
KH(°dH)	14	25	23	26	18	27	28	30	9	12	21	19
GH(°dH)	15	14	13	14	22	20	25	21	9	11	11	12
pH	6.6	7.4	8	8.0	8	7.8	8	7.8	7.4	7.0	7.4	7.6
NH_4(mg/L)	10	0.2	0.1	0.4	0.05	0.1	0.1	5	0.05	0.1	0.4	0.05
NO_2(mg/L)	0.01	<0.01	0.05	<0.01	0.025	<0.01	0.2	0.2	0.01	0.8	0.2	0.01
NO_3(mg/L)	0.5	<0.5	1	<0.5	1	<0.5	5	5	1	15	<0.5	0.5
PO_4(mg/L)	0.02	1.8	1.8	1.8	0.02	1.8	1.8	1.8	0.02	1.8	1.8	0.02
SiO_2(mg/L)	3	0.8	0.2	0.8	0.1	<0.1	0.2	0.2	0.1	3	0.2	0.1
Fe(mg/L)	0.05	0.05	<0.02	0.05	1	0.05	<0.02	0.1	0.02	<0.02	0.05	0.8
Cu(mg/L)	0.05	<0.05	<0.05	<0.05	0.05	<0.05	<0.05	<0.05	0.05	<0.05	<0.05	0.05
O_2(mg/L)	10	8	10	8	6	10	8	8	10	10	8	10

M^P - aquatic environment represented by bottled water; M^A - the aquatic environment from farm A; M^B - the aquatic environment from farm B; Mc - the aquatic environment from which specimens C_1 and C_2 originate; , and - stand for the initial values for the water from A and B farm and the bottled water.

Specimen A_1 was in M^A water, its own aquatic environment, for 8 days, and at the first signs of lethargy (loss of balance, leaning to one side, etc.), it was removed from the studied aquatic environment and its parameters of interest were analysed (Tables 4 and 5). Specimen B_2, released into the aquatic environment M^A, in addition to the stress factor of retention under laboratory conditions, was also subjected to the stress caused by the modification of the

environment. The individual survived in the water from farm A for 11 days, intervening in the parameters of interest (Figure 2), balancing NH_4 and implicitly the nitrite and nitrate values. The individual consumed Fe, Cu and SiO_2, balancing the values of these parameters. By comparing the values of M_i and $M_F^A(B_2)$, we notice that B_2 brought the parameters closer to the values of its own aquatic environment, with major changes being recorded for KH (total hydrogen carbonate concentration), pH, NH_4 (ammonia), PO_4 (phosphate content), and SiO_2 (silicate content).
The KH value increased by 9 units, also exceeding the value of the environment of origin by 5 units. KH, together with carbonates and CO_2, form a buffer system that prevents fluctuations in the pH of the aquatic environment, and it can be seen that the pH has been adjusted to the pH value of the aquatic environment of origin, at 8.
The value of NH_4 decreased considerably, by 9.9 units, which is noteworthy, considering that ammonia has a high degree of toxicity for aquatic organisms (Bud et al., 2010). The power of the individual to reduce the value of NH_4 can be explained by the high degree of adaptability of the species, but which was enhanced by the activities carried out on the farm where it comes from, where natural methods of developing the biological material were applied.
PO_4 (phosphate content), which has as its source "the general bio-geo-chemical circuit (mineralization of organic matter) including the processes of secretions, excretions, and cell lysis" (Bud et al., 2010), the other sources being irrelevant in laboratory (aquarium) conditions, increased up to 1.8 through the exchange of the specimen with the external environment. This fact emphasizes that the individual influenced the values of the water parameters, adjusting them according to his own needs.
Specimen B_2 was studied for 11 days. Showing signs of lethargy, it was removed from the water, and the final parameters of the aquatic environment were analysed (Tables 4 and 5).
Following the changes recorded in the composition of the water from A farm, it can be seen that the parameters that underwent major changes are KH, NH_4, PO_4, and SiO_2, while the other values were slightly influenced or not at all (Figure 3).

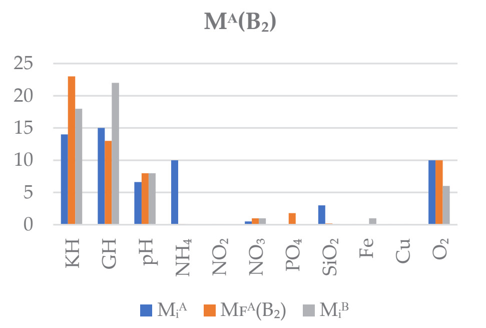

Figure 2. Average values of M^A parameters with B_2 fish

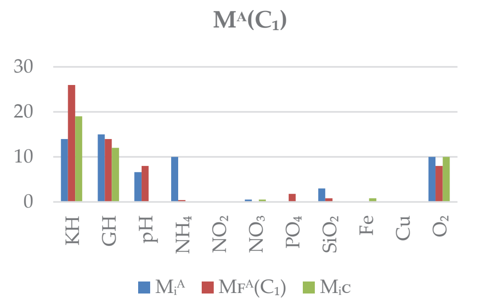

Figure 3. Average values of M^A parameters with C_1 fish

KH contributes to the formation of the buffer system that prevents fluctuations in the pH of the aquatic environment, and it can be seen that the pH did not exceed the maximum value up to which the carp develops within normal limits (7.7-9.00). However, it was modified by 1.4 units, exceeding the value of the water from which individual C_1 originated.
The value of NH_4 also decreased considerably in this case by 9.06 units, having the same explanation as in the previous case.
Analysing the values of the parameters of the water from which specimen C_1 comes, it is observed that the tendency of the changes made to the aquatic environment in which it was released is to bring the values as close as possible to those of its own environment. Thus, NO_2, NO_3, and Cu were brought to the values of the aquatic environment of origin.
The same PO_4 content as in the cases of specimens A_1 and B_2 was also recorded here, appearing to be a standard modification applied by different individuals to the same environment.

As for the value of the O_2 content, it was 2 units lower compared to the initial values of the aquatic environments studied, and of the aquatic environment of origin, but also of the aquatic environment in which it was released, demonstrating that the individual used a large amount of oxygen in the adaptability process. This fact is also supported by the aeration applied in the aquarium.
Specimen C_1 spent 16 days in the aquatic environment M^A, modifying the aquatic environment parameters recorded in Tables 4 and 5. At the first signs of apathy, the individual was removed from the water, and the parameters of the water in which it was released during this period were analysed.
It is observed that the pH is perfectly balanced at the value of 7.8, the KH value has increased, helping to limit pH fluctuations (Figure 4). Coming from an environment with an NH_4 content greater than 10 units, a slight increase in the level of this parameter is also observed in the aquatic environment where the specimen was released, and the increased value of phosphate, PO_4, demonstrates the individual's intervention on the environment. It should be emphasized that the change in PO_4 was limited to the value of 1.8 units, as in the case of the previously analysed aquatic environments. The theory is increasingly taking shape that the intervention of a specimen in the environment in which it is released changes the phosphate value to this level.
In this case, the A_2 specimen spent 9 days in the M^B environment, and at the first signs of apathy, it was removed from the water. The analysis of the water parameters in the aquatic environment M was carried out immediately after the extraction of the individual from the water (Tables 4 and 5).

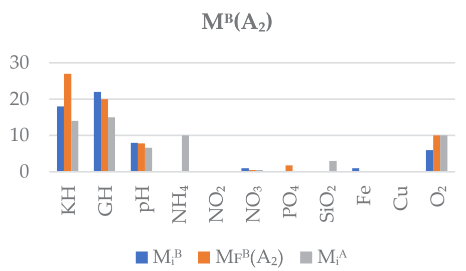

Figure 4. Average values of M^B parameters with A_2 fish

As in the previously presented situation of specimen A_1 that did not change the aquatic environment, in this case, also, the specimen that was released into water M^B, was only retained in laboratory (aquarium) conditions. The change in parameters was also interpreted as a response to stress stimuli.
The pH remained unchanged, NH_4 increased to the value of 0.1 mg/l, implicitly the values of NO_2 and NO_3 also increased (Figure 5), but the limits recommended in aquaculture were not exceeded.

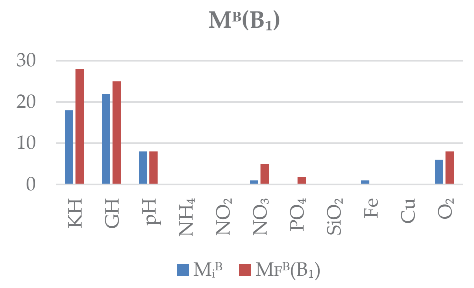

Figure 5. Average values of M^B parameters with B_1 fish

It is observed the value of the phosphate content increased up to 1.8 mg/l as in the previous cases.
Specimen B_1 was in the aquatic environment M^B, its environment of origin, for a duration of 8 days, and at the first signs of lethargy, it was removed from the water and the parameters of interest were analysed (Tables 4 and 6).
Coming from the same environment as specimen C_1, C_2 showed the same tendencies to influence the water in which it was released. In the aquatic environment from B farm, having the values of the analysed parameters close to those of the parameters of the aquatic environment of the individual's origin, the O_2 value increased by 2 units (Figure 6), showing that C_2 did not make as much effort to adapt as the specimen C_1. However, the Fe content decreased from the value of 1 to 0.1, the optimal concentration in water is up to 1 mg/l (Bud et al., 2010), and the Cu parameter value stabilized below 0.05 mg/l, like that of the aquatic environment of origin, thus describing an intervention of the specimen on the new environment. Copper dissolved in water is readily absorbed by fish, but a concentration of copper sulfate greater than 0.8 mg/l can lead to chronic toxicity in numerous

species (Hepher, 1988; Barnabé, 1991; Macovei, 2008; Momeu et al., 2018).
KH was the parameter that increased its value the most, by 12 units, but contributing, together with carbonates and CO_2, to the creation of the buffer system that prevents pH fluctuations, the pH level fell perfectly between the value of the environment of origin and that of the new environment, stabilizing at 7.8, the specimen showing a high degree of adaptability.
The data is performed in Tables 4 and 6.

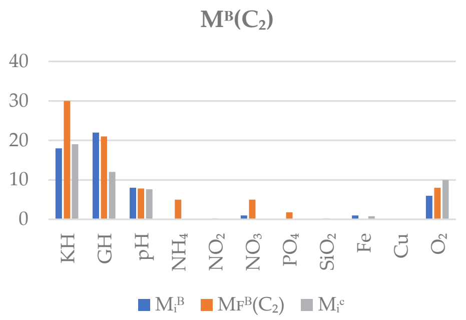

Figure 6. Average values of M^B parameters with C_2 fish

In this case, specimen A_3 was released in 100 l of still bottled water. Only 36 h after its release in the new environment, the individual presented physiological parameters incompatible with life, however, affecting the aquatic environment, significantly changing the values of NH_4, NO_2, NO_3, PO_4 and SiO_2 (Figure 7).

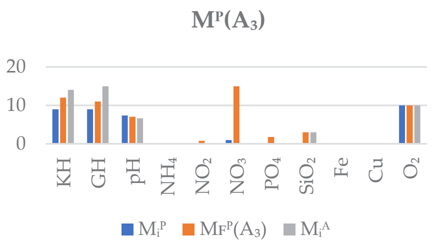

Figure 7. Average values of M^P parameters with A_3 fish

Coming from the aquatic environment M_i^A, where the value of NH_4 is greater than 10 units, it is observed that, at the end of the experiment, the value of this parameter also increased in the water in which it was released. Implicitly, the NO_2 and NO_3 values also increased, the nitrite value being in the range of 0.5-1 mg/l, which describes, depending on the sensitivity of each species, fatal values. *Cyprinus carpio* is a species with a high degree of adaptability, but in the study, the value of 0.8 mg/l of nitrite proved to be fatal.
During the short period spent in bottled water, the specimen also affected the pH by balancing it between the pH value of the water from which it came, M_i^A, and the pH value of the bottled water in which it was released, M_i^P, namely at the value 7.
The value of the silicate content increased up to 3 units, equaling that of the aquatic environment of origin, and the phosphate value did not deviate from the rule observed so far in the experiment and increased to 1.8 units as in the previous cases.
The data is highlighted in Tables 4 and 6.
In this situation, specimen B_3 was released in 100 l of still water. Coming from the M_i^B environment with values close to those of the water in which it was released, the individual spent 10 days in the new environment, during which time it intervened very little on the water parameters of interest.
However, it does not deviate from the rule that was observed in all specimens, increasing the phosphate value to 1.8 units. Coming from an environment with a water hardness higher than that of the new environment, B_3 contributed very little to the modification of NH_4 values, implicitly NO_2 and NO_3, but also SiO_2, Fe and Cu (Figure 8, Tables 4 and 7).

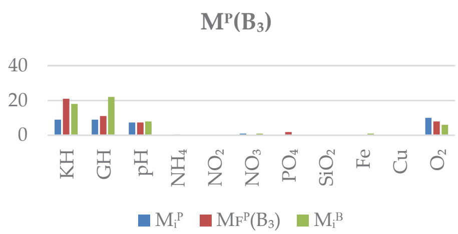

Figure 8. Average values of M^P parameters with B_3 fish

Discussions
The released specimens in the water from A farm survived in the conditions created in the laboratory (in an aquarium with 100 l of water) for 8, 10, and 16 days, respectively. It is observed that the water hardness increased supporting the pH stability (Figures 1, 2, 3).

The initial value of the NH_4 content was over 10 units and, in each situation, the individuals managed to stabilize it at values much closer to those of the aquatic environments from which they came, except for specimen A_1, which was not released in a new aquatic environment, but which brought significant changes to the water. The change in parameters was interpreted as a response to stress stimuli. The individual, due to the growth technologies applied in the farm and the extent of the accumulation surface from which it originates, is classified as wild, and the reduction of the movement surface to 100 l of water represented a strong stress factor.
The consumption of Fe, Cu and SiO_2, together with the fluctuations recorded in the O_2 values, demonstrate the efforts made by each specimen to adapt, by creating the aquatic environment conducive to survival.
Under conditions created by the aquatic environment of B farm (Figures 4, 5, 6), B_1 and C_2 specimens brought more "bold" changes. Each individual deviated from the values of the parameters studied, without exceeding the limits recommended in aquaculture, and the phosphate content, as in the aquatic environment case of A farm, was fixed at the value of 1.8 mg/l by each individual, marking the degree of intervention on water.
In the aquatic environment represented by still bottled water (Figures 7, 8), specimen A_3, which came from water with NH_4 content value above 10 mg/l, survived only 36 h, bringing, however, changes to the aquatic environment in which it was released. The pH was balanced at the value of 7, and the value of the SiO_2 content increased to the value of the aquatic environment of origin, but the value of the nitrite content increased the toxicity of the water to the fatality of the individual.
Sample B_3, however, which came from an aquatic environment with values of water parameters close to those of still bottled water did not bring considerable changes to the water, but survived 10 days in the aquatic environment under study. The consumption of O_2 demonstrates the effort made by the individual in the adaptability process.
Applying the standard statistical tests, it was possible to observe the differences between the aquatic environments and the influences brought to them by each fish. Thus, in Table 4 and Table 7 it can be observed that for the GH and Cu parameters, there were no influences of the analysed factor (i.e., the farm or the type of water) on the water quality. However, in the vast majority of cases, there were differences between the averages for the other parameters (such as NH_4, phosphate, and SiO_2) between the analysed groups. Thus, it can say that each fish brought significant changes to the water quality in the environment where it was released.

Table 4. Fisher Tests for studied aquatic environments

Aquatic environment	M_A			M_B			M_P	
Fish / Parameter (unit)	A_1	B_2	C_1	A_2	B_1	C_2	A_3	B_3
KH (°dH)	***			***			***	
GH (°dH)	ns			***			***	
pH	***			***			***	
NH_4 (mg/L)	***			***			***	
NO_2 (mg/L)	***			***			***	
NO_3 (mg/L)	***			***			***	
PO_4 (mg/L)	***			***			***	
SiO_2 (mg/L)	***			**			***	
Fe (mg/L)	***			***			***	
Cu (mg/L)	ns			ns			ns	
O_2 (mg/L)	***			***			***	

*** $\hat{F} > \hat{F}0.1\%$ - very significant differences between samples
** $\hat{F}1\% < \hat{F} < \hat{F}0.1\%$ - distinctly significant differences between samples
ns $\hat{F} < \hat{F}5\%$ - insignificant differences between samples

Table 5. Tukey Tests for A farm aquatic environment

Specification	- C_1	- A_1	- B_2	B_2- C_1	A_1- B_2	A_1- C_1
KH (°dH)	***	***	***	**	ns	ns
pH	***	***	***	***	***	***
NH_4 (mg/L)	***	***	***	***	***	***
NO_2 (mg/L)	***	ns	ns	***	ns	***
NO_3 (mg/L)	***	***	***	ns	ns	ns
PO_4 (mg/L)	***	***	***	ns	ns	ns
SiO_2 (mg/L)	***	***	***	***	***	ns
Fe (mg/L)	***	***	***	ns	ns	ns
O_2 (mg/L)	***	***	ns	***	***	ns

*** w > w1% - very significant differences between samples
** w5% < w < w1% -significant differences between samples
ns w < w5% - insignificant differences between samples

In the case of B farm aquatic environment, in addition to the parameters GH and Cu, it is also observed for other parameters such as SiO_2 and NH_4, respectively, that there are no significant differences between the majority of the groups, as detailed in Table 6.

Table 6. Tukey Tests for B farm aquatic environment

Specification	- A_2	- B_1	- C_2	A_2 - B_1	A_2 - C_2	B_1 - C_2
KH (°dH)	***	***	***	ns	ns	***
pH	***	***	***	ns	***	***
NH_4 (mg/L)	***	***	***	ns	ns	ns
NO_2 (mg/L)	***	***	***	***	*	***
NO_3 (mg/L)	***	***	ns	***	***	***
PO_4 (mg/L)	***	***	***	ns	ns	ns
SiO_2 (mg/L)	***	***	ns	ns	***	***
Fe (mg/L)	***	ns	***	**	***	***
O_2 (mg/L)	***	***	***	ns	***	***

*** difference between averages > w1%, very significant differences between samples
** w5% < difference between averages < w1%, significant differences between samples
ns difference between means < w5%, insignificant differences between samples

For bottled water, we notice that the factor influences water quality for almost all parameters, the only exception being Cu, as represented in Table 7.

Table 7. Tukey Tests for bottled water environment

Specification	- A_3	- B_3	A_3- B_3
KH (°dH)	***	***	***
GH (°dH)	***	***	***
pH	ns	ns	***
NH_4 (mg/L)	ns	***	***
NO_2 (mg/L)	***	***	ns
NO_3 (mg/L)	***	ns	ns
PO_4 (mg/L)	***	***	ns
SiO_2 (mg/L)	***	ns	ns

*** difference between the averages > w1%, very significant differences between samples
** w5% < difference between averages < w1%, significant differences between samples
ns difference between means < w5%, insignificant differences between samples

After applying the tests, the intervention of each individual in the aquatic environment that they inhabit is observed. These results are relevant in the context of good practices in fish farms, contributing to the application of good practices in aquaculture in order to optimize them economically.

CONCLUSIONS

The results showed that the carp specimens were able to adapt to the new environment by bringing changes to the water parameters. The study found that NH_4 (ammonia), PO_4 (phosphate content), SiO_2 (silicate content), and KH (total hydrogen carbonate concentration) were the parameters that underwent significant changes due to the carp's intervention. The carp specimens were able to bring the parameters closer to the values of their own aquatic environment, which indicates their adaptability. The study also found that PO_4 was a standard modification applied by different individuals to the same environment. The study highlights the importance of monitoring the water parameters in aquaculture and how *C. carpio*'s intervention affects these parameters.

The next research direction could focus on identifying the genetic and physiological factors that allow carp to adapt to new environments and intervene in the aquatic environment. Research could also explore the potential of carp to improve water quality and mitigate the impact of pollution on aquatic ecosystems. Another possible research direction could investigate the potential of carp's intervention in aquaculture for improving fish health and production.

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NEW DATA ON THE HELMINTH FAUNA OF *Alosa immaculata* Bennett, 1835 FROM THE BULGARIAN SECTION OF THE DANUBE RIVER, NORTHWESTERN BULGARIA

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Abstract

During 2019-2021, six specimens of pontic shad (Alosa immaculata Bennett, 1835); family Clupeidae were examined for parasites. The specimens were collected from three biotopes (Kudelin, Yasen, and Koshava) located in the section of the Danube River in the northwestern part of Bulgaria. Infection with 3 species of helminths was found – 1 species of the class Trematoda (Lecithaster confusus Odhner, 1905); 1 species of the class Acanthocephala (Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908) and 1 species of the class Nematoda (Hysterothylacium gadi aduncum (Rudolphi, 1802) Deardorff et Overstreet, 1981 (larvae)). Ecological indices of the found helminth species were examined. The purpose of the study is to provide new data on the helminth fauna of the pontic shad from the freshwater ecosystem of the Danube River in Bulgaria. Al. immaculata is a new host for one endohelminth species (L. confusus). Two of the investigated biotopes (Koshava and Yasen) are new habitats for the found endohelminths in pontic shad.

***Key words**: helminths, Koshava, Kudelin, pontic shad, Yasen.*

INTRODUCTION

Pontic shad (*Alosa immaculata* Bennett, 1835) is a passage fish, subject to commercial fishing, and inhabits the Black Sea and the Sea of Azov. The species perform breeding migrations. It spawns in the Danube, Dnieper, Dniester, Don rivers, and others during the period April-August (Karapetkova & Zhivkov, 2006; Visnjic-Jeftic et al., 2010; Đikanović et al., 2018; Smederevac-Lalić et al., 2018; Fröse & Pauly, 2022). The species does not feed during migrations (Ciolac, 2004). The diet of the pontic shad includes fish and crustaceans (Golemanski, 2011; Fröse & Pauly, 2022). Water pollution, overfishing, loss and fragmentation of habitats, construction of dams, low water levels, etc. have a negative impact on the number of species (Golemanski, 2011; Smederevac-Lalić et al., 2018; Grecu et al., 2020; Fröse & Pauly, 2022). Due to the construction of Iron Gate I and Iron Gate II, today the species is found in the Danube River up to the confluence of the Timok River (Visnjic-Jeftic et al., 2010; Smederevac-Lalić et al., 2018). The parasite fauna of the pontic shad from the Bulgarian section of the Danube River is poorly studied (Kakacheva-Avramova et al., 1978; Kirin et al., 2013; Nachev et al., 2022). Few authors provide data on the parasite fauna of *Al. immaculata* (Đikanović et al., 2018; Grecu et al., 2020; Stroe et al., 2021; Stroe et al., 2022). The purpose of the present study is to provide new data on the helminth fauna of *Al. immaculata* from the freshwater ecosystem of the Danube River in Northwestern Bulgaria.

MATERIALS AND METHODS

Six specimens of *Al. immaculata* from three locations (presented as biotopes) from the Bulgarian section of the Danube River were subjected to parasitological examination. Kudelin biotope (44°12'07.9"N, 22°41'28.2"E), Yasen biotope (44°07'26.6"N, 22°52'42.1"E) and Koshava biotope (44°03'59.9"N, 23°02'10.2"E) are located on the right bank of the river, in Vidin Province, Northwestern Bulgaria (Figure 1).

The fish were caught with fishing gear according to permits issued by the Executive Agency for Fisheries and Aquaculture (EAFA). The scientific name of the species was given by Karapetkova & Zhivkov (2006); Fröse & Pauly (2022). Each caught fish specimen was weighed and measured (Table 1).

Figure 1. Location of biotopes from the Bulgarian section of the Danube River, Northwestern Bulgaria (https://www.google.bg/maps/place/Видин)

Table 1. Metric data (total body length (TL; in centimeters); maximum body height (MH; in centimeters); body weight (BW; in grams) of the studied specimens of *Alosa immaculata*

Danube River		TL (cm)	MH (cm)	BW (g)
Alosa immaculata **N = 6**	**min-max**	9.8-30	2.1-5.5	7-146
	Mean±SD	20.20±11.33	3.66±1.71	63.60±73.93

The parasitological examination of the caught specimen's pontic shad was carried out according to standard methods (Zashev & Margaritov, 1966). All found helminth specimens were isolated and stored in 70% ethyl alcohol for further processing. Permanent microscopic preparations were made from the representatives of class Trematoda, and temporary microscopic preparations were made from the representatives of classes Acanthocephala and Nematoda (Dubinina, 1948; Zashev & Margaritov, 1966; Moravec, 2013). The type of all found helminths was determined (Zashev & Margaritov, 1966; Moravec, 2013; and others). Basic ecological indices were calculated and presented: mean intensity (MI); mean abundance (MA) and prevalence (P%) (Bush et al., 1997).

RESULTS AND DISCUSSIONS

Model fish species

Al. immaculata is a pelagic-neritic fish. The species winters in the Black Sea. The fish have a weight of up to 1 kg and a body length of up to 40 cm (Ciolac, 2004). They live up to 7-8 years (Karapetkova & Zhivkov, 2006; Fröse & Pauly, 2022). Pontic shad is protected by national and international legislation. The species is included in the Red Book of the Republic of Bulgaria with the category "VU=Vulnerable", in the Biological Diversity Act, and also in the IUCN Red List with the category "VU=Vulnerable", in the Bern Convention and in the Habitats Directive (Convention on the conservation of European wildlife and natural habitats, 1982; Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora, 1992; Biological Diversity Act, 2002; Freyhof & Brooks, 2011; Golemanski, 2011).

Ecologohelminthological examination

For the period 2019-2021, a total of 6 specimens pontic shad were examined for parasites – two specimens from each of the three biotopes (Kudelin, Koshava, Yasen). Infection with a total of 3 endohelminth species was found: *Lecithaster confusus* Odhner, 1905 (class Trematoda); *Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908 (class Acanthocephala) and *Hysterothylacium gadi aduncum* (Rudolphi, 1802) Deardorff et Overstreet, 1981 (larvae) (class Nematoda) (Table 2).

Table 2. Taxonomic position, localization, biotopes, season of detection of *Lecithaster confusus*, *Pomphorhynchus laevis*, and *Hysterothylacium gadi aduncum*

Helminth species	***Lecithaster confusus* Odhner, 1905**	***Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908**	***Hysterothylacium gadi aduncum* (Rudolphi, 1802) Deardorff et Overstreet, 1981**
Taxonomic position	CLASS TREMATODA RUDOLPHI, 1808 Family Lecithasteridae Skrjabin et Guschanskaja, 1954 Genus *Lecithaster* Lühe, 1901	CLASS ACANTHOCEPHALA (RUDOLPHI, 1808) Family Pomphorhynchidae Yamagiti, 1939 Genus *Pomphorhynchus* Monticelli, 1905	CLASS NEMATODA RUDOLPHI, 1808 Family Raphidascarididae Genus *Hysterothylacium* Ward & Magath, 1917
Localization	intestine	intestine	intestine
Biotope	Koshava	Koshava, Yasen	Koshava
Season	spring	spring, summer	spring

In the present study, one helminth species was common to pontic shad from two of the studied biotopes (Koshava and Yasen). The species diversity of the found helminths (3 species) was largest in pontic shad from Koshava biotope, followed by Yasen biotope (1 species). The specimens from Kudelin biotope were not infected. Only one of the examined specimens from Koshava biotope was infected, and 3 endohelminth species were found. Of them, *H. aduncum* had the highest mean intensity and mean abundance (MI = 7.00; MA = 3.50), while all three helminth species had the same prevalence (P% = 50.00). Both examined pontic shad specimens from Yasen biotope were infected with one helminth species - *P. laevis*. This helminth species is common to *Al. immaculata* from Koshava and Yasen biotopes. *P. laevis* had the same mean intensity in both biotopes (MI = 1.00), but higher mean abundance and prevalence in Yasen biotope (MA = 1.00; P% = 100.00) (Table 3).

Table 3. Species diversity and ecological indices in the helminth community of *Alosa immaculata* from the Danube River

***Alosa immaculata* (N = 2 / Koshava) Parasite species**	**n**	**p**	**MI**	**MA**	**P%**	**R**
Lecithaster confusus Odhner, 1905	1	1	1.00	0.50	50.00	1
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	1	1	1.00	0.50	50.00	1
Hysterothylacium gadi aduncum (Rudolphi, 1802) Deardorff et Overstreet, 1981 (larvae)	1	7	7.00	3.50	50.00	7
***Alosa immaculata* (N = 2 / Yasen) Parasite species**	**n**	**p**	**MI**	**MA**	**P%**	**R**
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	2	2	1.00	1.00	100.00	1

N - number of investigated fish; n - number of infected fish; p - number of fish parasites; MI - mean intensity; MA - mean abundance; P% - prevalence; R - range

The first intermediate host of *L. confusus* is the snail *Odostomia trifida* (Totten, 1834), and the second host is the crustaceans *Acartia clausi* Giesbrecht, 1889; *Centropages hamatus* (Lilljeborg, 1853). Definitive hosts are *Clupea harengus* Linnaeus, 1758; *Al. immaculata*; *Al. tanaica* (Grimm, 1901); *Clupeonella cultriventris* (Nordmann, 1840); *Atherina boyeri*

Risso, 1810; *Ath. hepsetus* Linnaeus, 1758, others (Bykhovskaya-Pavlovskaya et al., 1962; Gaevskaya et al., 1975; Kakacheva-Avramova, 1983; Bauer (Ed.), 1987). Definitive hosts of *P. laevis* are fish from the families Cyprinidae, Salmonidae, Percidae, Siluridae, etc. The intermediate host of the species is *Gamarus pulex* (Linnaeus, 1758) (Petrochenko, 1956; Kakacheva-Avramova, 1983; Bauer (Ed.), 1987). Typical species of *H. aduncum* are *Alosa alosa* (Linnaeus, 1758); *Alosa fallax* (Lacepède, 1803); *Cl. harengus*. In Europe, the species was reported for the following migratory freshwater fish species: *Al. immaculata*; *Salmo trutta* Linnaeus, 1758; *Salmo salar* Linnaeus, 1758; *Oncorhynchus mykiss* (Walbaum, 1792); *Chondrostoma nasus* (Linnaeus, 1758); *Tinca tinca* (Linnaeus, 1758); *Phoxinus phoxinus* (Linnaeus, 1758); *Esox lucius* Linnaeus, 1758; *Perca fluviatilis* Linnaeus, 1758; others. Intermediate hosts are the marine copepods *Acartia bifilosa* (Giesbrecht, 1881) and *Eurytemora affinis* (Poppe, 1880) (Bauer (Ed.), 1987; Moravec, 2013).

P. laevis and *H. aduncum* found in the present study were reported for pontic shad from the Bulgarian section of the Danube River (Kakacheva-Avramova et al., 1978 and Kirin et al., 2013; Nachev et al., 2022, respectively). *L. confusus* was reported for *Al. immaculata* from the Black Sea (Özer et al., 2013; Sezgin et al., 2017). Đikanović et al. (2018) reported *Contracaecum* sp. in pontic shad from the Danube River (861 river km) in the region of Prahovo, Serbia, and *Contracaecum* sp., *Contracaecum siniperca* Dogiel & Achmerov, 1946 and *Contracaecum bidentatum* (Linstow, 1899) Skrjabin, 1917 in pontic shad from the Danube delta, Romania. Grecu et al. (2020) studied pontic shad from the Romanian section of the Danube River, in the region of Cotul Pisicii and reported four species of parasites - *Mazocreas alosae* (Hermann, 1782); *Allocreadium isoporum* (Looss, 1894); *Nicolla skrjabini* (Iwanitzky, 1928) Dollfus, 1960 (syn. *Coitocoecum skrjabini* (Ivanitzky, 1928)); *Hysterothylacium aduncum* (Rudolphi, 1802) (syn. *Contracaecum aduncum* (Rudolphi, 1802)). The authors indicated that *H. aduncum* (a marine parasite species) had the highest prevalence and mean intensity (P% = 94.44, MI = 55.76 ± 7.65 in 2011; P% = 95.45, MI = 32.38 ± 6.88 in 2018), which is related to the diet of the pontic shad (a migratory species), a diet consisting of intermediate for the nematode crustaceans and fish hosts. *H. aduncum* and *M. alosae* were reported in pontic shad from the Romanian section of the Danube River - in the region of Moldova Nouă (1,048 river km) and Giurgiu (493 river km) (Stroe et al., 2021) and in the section between 169 and 197 river km (Stroe et al., 2022) (Table 4).

Table 4. Distribution of the found helminths (in the present study) of *Alosa immaculata* from the Danube River and its basin

Biotopes / **Helminth species**	Kudelin biotope	Koshava biotope	Yasen biotope	Danube River in other countries	Danube River Basin in other countries	Black Sea Basin	Danube River in Bulgaria	Danube River Basin in Bulgaria
Lecithaster confusus	-	+	-	-	-	+	-	-
Pomphorhynchus laevis	-	+	+	-	-	-	+	-
Hysterothylacium aduncum	-	+	-	+	-	-	+	-

CONCLUSIONS

During the helminthological examination of six specimens pontic shad collected from three biotopes (Kudelin, Yasen, Koshava), infection with 3 helminth species belonging to classes Trematoda, Acanthocephala, and Nematoda was found. The highest mean intensity; mean abundance and range were found for *Hysterothylacium gadi aduncum* (Rudolphi, 1802) Deardorff et Overstreet, 1981 (MI = 7.00; MA = 3.50; R = 7) of *Al. immaculata* from Koshava biotope. The highest prevalence had *Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908 of *Al. immaculata* from Yasen biotope (P% = 100.00). The pontic shad is reported for the first time as a host of the trematode *L. confusus* from the Danube River and the river basin, including on Bulgarian territory. The studied biotopes are new habitats for the found endohelminths of *Al. immaculata*.

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HELMINTHS AND HELMINTH COMMUNITIES OF *Perca fluviatilis* (Linnaeus, 1758) AND *Vimba melanops* (Heckel, 1837) FROM MARITSA RIVER, BULGARIA

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Abstract

As a result of the study of 10 specimens Perca fluviatilis Linnaeus, 1758 and 10 specimens Vimba melanops (Heckel, 1837) from the Maritsa River, Aegean water basin, Bulgaria, infection with 5 helminth species are presented (Allocreadium isoporum (Loos, 1894); Proteocephalus percae (Müller, 1780); Caryophyllaeus laticeps (Pallas, 1781); Contracaecum sp., larvae and Acanthocephalus lucii (Müller, 1776)). All identified parasite species are autogenous to the parasite communities of the perch and Macedonian vimba, except Contracaecum sp.*, which is an allogeneic species. Infection indices are discussed and pathways of helminth flux circulation are traced. The dominant structure of the helminth communities was analyzed. New data on the helminths and their communities in the two species of freshwater fish, as well as on the ecological status of the studied biotopes of the freshwater ecosystem are presented.*

Key words*: Aegean water basin, ecological status, helminth communities, Macedonian vimba, perch*.

INTRODUCTION

The Maritsa River, Aegean water basin, springs from the territory of Bulgaria and is one of the longest rivers (472 km, of which 322 are on Bulgarian territory) in the country, after the rivers Danube, Iskar, and Tundzha. The river begins at 2378 meters above sea level in the Rila Mountain, from the two Marichini lakes, located between the peaks of Mancho (2771 m, a.s.l.), Marishki chal (2765 m a.s.l.) and Bliznacite (2779 m a.s.l.). The Maritsa River flows into the Aegean Sea (Valkanov, 2000). The rapidly increasing anthropogenic impact disrupts the ecological balance in nature. Freshwater ecosystems are particularly sensitive to negative impacts. Maritsa river is also under the such influence (pollution from industry, agriculture, household, etc.). The river is a habitat for many valuable and globally protected animals and plants. The Maritsa River has the role of a hydroclimatic pathway for the penetration of Mediterranean and sub-Mediterranean influence, which favors the presence of rich flora and fauna. Along the river, protected areas BG0000578 "Maritsa River" have been declared under the Habitats Directive (Directive 92/43/EEC), as well as BG0002081 Maritsa-Parvomai and BG0002087 Maritsa-Plovdiv under the Birds Directive (Directive 79/409/EEC) as part of the National and Ecological Network NATURA 2000 (https://natura2000.egov.bg/EsriBg.Natura.Public.Web.App).

Parasites are part of biological diversity. Parasitic organisms are considered to constitute the richest group of species on Earth. They testify to the state of the food chains in the respective habitats because most of them have complex development cycles associated with the participation of free-living organisms and intermediate hosts. Some parasites can accumulate heavy metals and other pollutants, which they biomagnify and transmit through food chains. Many studies have established both losses of fish resources and dangerous parasites for the ichthyofauna and humans but also the need for parasite conservation (Gómez & Nichols, 2013; Auld & Tinsley, 2015; Biswal, 2020; Carlson et al., 2020, etc.).

The study presents new data on the helminths and helminth communities of *Perca fluviatilis* (Linnaeus, 1758) and *Vimba melanosps* (Heckel, 1837), as well as data on the ecological status of the studied biotopes.

MATERIALS AND METHODS

A total of 10 specimens *Perca fluviatilis* Linnaeus, 1758 and 10 specimens *Vimba melanosps* (Heckel, 1837) were examined for helminths. The fish are caught with fishing rods from the river Maritsa in the vicinity of the village of Milevo. The names of the fish are presented according to the FishBase database (Fröse & Pauly, 2022). Helminthological examinations are carried out by the methods described by Petrochenko, 1956; Zashev & Margaritov, 1966; Bauer (Ed.), 1987; Moravec, 2013, etc. Helminth specimens were fixed and preserved with 70% ethyl alcohol in Eppendorf tubes. For the specimens of classes Trematoda and Cestoda, the methods of Georgiev et al., 1986; Scholz & Hanzelová, 1998 were used and for classes, Acanthocephala and Nematoda – the methods of Zashev & Margaritov, 1966; Moravec, 2013 were used. Analyses of the helminth community structure have been performed in both levels: infracommunity (total and mean number of species; total and mean number of specimens; Brillouin's index of diversity - HB) and component community (prevalence (P%) and mean intensity (MI) for each species) (Bushatal, 1997; Kennedy, 1993, 1997; Magurran, 1988). The species are divided into core species (P%>20), component species (P%>10), and accidental species (P%<10) (Kennedy, 1993). The diversity measures are calculated by software products Statistica 10 (StatSoft Inc., 2011) and MS Excel (Microsoft 2010).

RESULTS AND DISCUSSIONS

Model fish species

Macedonian vimba (*Vimba melanops* (Heckel, 1837); Cyprinidae) is a freshwater, brackish, and demersal fish that inhabits rivers of the Aegean watershed (Vardar, Pinios, Struma, Maritsa, etc.) and its tributaries. The species mainly prefer the middle and less often the lower reaches of rivers with sandy and gravelly bottoms. *V. melanops* reproduces during the period of May-June with characteristic migrations to the upper courses of the rivers, reaching sexual maturity at 2-3 years. Macedonian vimba spawns in stony and gravelly places. It feeds mainly on benthic invertebrates and algae. It reaches a maximum of 30-40 cm in length (Kottelat & Freyhof, 2007; Zhivkov & Karapetkova, 2009). The species appears in the IUCN Red list with the category Data Deficient (DD, IUCN) (Froese & Pauly, 2022). *V. melanops* is included in the Red Book of Bulgaria, volume 2. Animals, with a nature conservation status of vulnerable (VU, IUCN) (Golemanski (Ed.), 2011). *V. melanops* is an endemic species to the Aegean watershed (Kolev, 2013).

Perca fluviatilis Linnaeus, 1758 (Percidae) is a freshwater, brackish, and demersal fish species, which inhabits freshwater ecosystems in the temperate and subtropical zones in North America and Eurasia. Perch can be found in rivers, streams, and lakes in almost all of Europe. It is a typical predator. Larvae feed on zooplankton and adults feed on larvae of other fish species, insects, and small fish. The species lives in schools but is not a territorial fish. It hunts during the day. Males reach sexual maturity at 2-3 years, and females at 3-6 years. The perch reproduces in the period April-May, at a temperature not lower than 7-8°C (Kottelat & Freyhof, 2007; Zhivkov & Karapetkova, 2009). The species appears in the IUCN Red list with the category Least Concern (LC, IUCN) (Froese & Pauly, 2022).

Helminths and helminth community structure

As a result of the ecologoparasitological examinations of 10 specimens of perch (*P. fluviatilis*) and 10 specimens of Macedonian vimba (*V. melanops*), infection with 5 taxa of helminths was found: *Allocreadium isoporum* (Loos, 1894); *Proteocephalus percae* (Müller, 1780); *Caryophyllaeus laticeps* (Pallas, 1781); *Contracaecum* sp., larvae; *Acanthocephalus lucii* (Müller, 1776). Found helminth species belong to classes Trematoda (1) and Cestoda (2); Nematoda (1) and Acanthocephala (1), respectively. Macedonian vimba is represented by a bigger number of helminth species in helminth communities (three helminth species) than perch (two helminth species).

The development of *All. isoporum* is carried out with the participation of two intermediate hosts - snails of the genus *Sphaerium* Scopoli, 1777 and insect larvae of the genera *Ephemera* Linnaeus, 1758, *Anabolia* Stephens, 1837, *Chaetopterix* Cuvier, 1827. Definitive hosts are mainly carp fish (Cyprinidae). Definitive hosts of *Pr. percae* mainly are *Gymnocephalus cernua* (Linnaeus, 1758), *P. fluviatilis* and *Esox lucius* Linnaeus, 1758, etc. Intermediate hosts are copepods from the genus *Cyclops* Müller, 1785. The development of *C. laticeps* is carried out with the participation of intermediate hosts *Tubifex tubifex* (Müller, 1774), *T. barbatus* (Grube, 1860), *Limnodrilus claparedeanus* Ratzel, 1868. Definitive hosts are freshwater fish from Cyprinidae, with specific hosts from the genus *Abramis* Cuvier, 1816. The intermediate host of *Ac. lucii* is *Asellus aquaticus* (Linnaeus, 1758) and definitive hosts are mainly freshwater fish species from Cyprinidae, rarely from Percidae, Siluridae, Salmonidae, Esocidae, Gadidae, Cobitidae, Anquillidae. Definitive hosts of *Contracaecum* sp. are fish-eating birds from *Ardea* Linnaeus, 1758, and *Nicticorax* Forster, 1817. Intermediate hosts are fish species from Cyprinidae, Percidae, Clupeidae, etc. (Petrochenko, 1956; Zashev & Margaritov, 1966; Kakacheva-Avramova, 1983; Bauer (Ed.), 1987; Moravec, 2013, etc.) (Table 1).

The intermediate hosts of established helminth taxa are detritophages (DF = deposit feeders) (Belkinova et al., 2013). The representatives of the genus *Cyclops* are bioindicators for β-α-mesosaprobity in examined habitats; *T. tubifex* and *T. barbatus* - for p-saprobity; *L. claparedeanus* - for p-α-mesosaprobity, and *A. aquaticus* is a bioindicator for α-mesosaprobity. In addition to being bioindicators of saprobity, some scientific studies show that these intermediate hosts are capable of accumulating heavy metals, which easily biomagnetize and reach higher organisms along the food chain, which poses a danger to freshwater fish and their consumers – fish-eating birds, humans, etc. (Ali & Fishar 2005; Maltby, 1991, etc.; Belkinova et al., 2013; Łuszczek-Trojnar et al., 2014).

Component community

Ac. lucii and *Contracaecum* sp. were infected examined fish hosts in 100%, respectively. The highest mean intensity in helminth communities of *V. melanosps* is distinguished for *Contracecum* sp., larvae (MI=8.5), followed by those of *C. laticeps* (MI=3.0) (Table 1).

Table 1. Biodiversity, mean intensity, the prevalence of parasites

Helminth species	Intermediate hosts	Definitive hosts: *Perca fluviatilis* Linnaeus, 1758 (N=10)		Definitive hosts: *Vimba melanops* (Heckel, 1837) (N=10)	
		P%	n/p Range MI	P%	n/p Range MI
Trematoda					
1. *Allocreadium isoporum* (Loos, 1894)	Mollusca, Insecta	-	-	10%	½ 2 2.0
Cestoda					
2. *Proteocephalus percae* (Müller, 1780)	Copepoda	30%	3/7 1-4 2.27	-	-
3.*Caryophyllaeus laticeps* (Pallas, 1781)	Oligocheta	-	-	50%	5/15 1-4 3.0
Nematoda					
4. *Contracaecum* sp., larvae	Crustacea, Copepoda, Cyprinidae	-	-	100%	10/85 1-16 8.5
Acanthocephala					
5. *Acanthocephalus lucii* (Müller, 1776)	Amphipoda	100%	10/28 1-4 2.8	-	-

Mean intensities of *Pr. percae* and *Ac. lucii* in helminth communities of perch are almost the same (MI=2.7 and MI=2.8, respectively) (Table 1). All established parasite species are autogenous to the parasite communities of *P. fluviatilis* and *V. melanops*, except for *Contracaecum* sp., which is an allogeneic species. The high mean intensity of infection with *Contracecum* sp. can lead to significant losses of fish resources as well as human health problems (Zashev & Margaritov, 1966; Zaharieva, 2022).

Infracommunity

Ten specimens of perch and ten specimens of Macedonian vimba were infected with one helminth species (100%). There are no uninfected fish specimens from both fish species. Macedonian vimba was infected with three species of helminth and also bigger specimens than perch (104 and 35 specimens, respectively). In perch, specimens infected with one species of helminth predominate (70%), while in Macedonian vimba, specimens infected with two types of helminth predominate (60%). Brillouin's indices of diversity are HB=0,45 (*P. fluviatilis*) and HB=0.48 (*V. melanosps*) (Table 2).

Table 2. Infracommunity data

Perca fluviatilis Linnaeus, 1758		
Number of helminth species		
Total number	2	
Number of infected fish	7	3
Number of helminth species	1	2
Number of helminth specimens		
Total number	35	
Mean ± SD	2.69±1.25	
Range	1-4	
Mean HB ± SD	0.45±0.29	
***Vimba melanops* (Heckel, 1837)**		
Number of helminth species		
Total number	3	
Number of infected fish	4	6
Number of helminth species	1	2
Number of helminth specimens		
Total number	104	
Mean ± SD	6.38±5.86	
Range	1-16	
Mean HB ± SD	0.48±0.31	

Discussions

P. fluviatilis helminths found in this study (*Pr. percae* and *Ac. lucii*) have been reported in previous studies on the freshwater ecosystem and perch (Table 3).

Table 3. Endohelminth species of *Perca fluviatilis and Vimba melanops* reported from other studies Aegean water basin, Bulgaria

Parasite species	Authors	Host (Locality)
***Perca fluviatilis* Linnaeus, 1758**		
Cestoda		
Proteocephalus percae (Müller, 1780)	Todorova-Traykova & Chunchukova, 2018	*P. fluviatilis* (rezervoir Batak)
	Kuzmanova et al., 2019	***P. fluviatilis*** **(river Maritsa)**
Acanthocephala		
Acanthocephalus lucii (Müller, 1776)	Margaritov, 1959	*Silurus glanis*, *Squalius cephalus* (r. Tundhza)
	Kuzmanova et al., 2019	***P. fluviatilis*** **(r. Maritsa)**
	Chunchukova et al., 2019	*Alburnus alburnus* (r. Maritsa)
***Vimba melanops* (Heckel, 1837)**		
Trematoda		
Allocreadium isoporum (Loos, 1894)	Kakacheva-Avramova, 1965	*Barbus cyclolepis*, *Gobio gobio*, *Squalius orphaeus* (rivers Syuyutlijka, Asenitsa, Bedechka, rez. Azmaka, rez. 40-te izvora)
	Margaritov, 1965	*Squalius orphaeus* **(r. Maritsa)**, *Barbus cyclolepis* (r. Vacha)
	Kirin, 2000, 2001	*Squalius orphaeus* **(r. Maritsa)**
	Kirin et al., 2019	*Squalius orphaeus* (r. Stryama)
	Kirin et al., 2020	*Barbus cyclolepis* (r. Tamrashka)
Cestoda		
Caryophyllaeus laticeps (Pallas, 1781)	Margaritov, 1959	*Alburnus alburnus* (r. Tundzha)

All. isoporum has been reported for the Maritsa River from other fish species, while *C. laticeps* and *Contracaecum* sp. were not reported for the Maritsa river. *V. melanosps* is reported for the first time as a host of *All. isoporum*, *C. laticeps* and *Contracaecum* sp. in Bulgaria. The helminth species found in this study have been reported also in other studies, mainly referring to the Danube basin: 1). *Pr. percae* of G*ymnocephalus schraetser* (Linnaeus, 1758) (syn. *Acerina schraetser*) *Sander volgensis* (Gmelin, 1789) (syn. *Stizostedion volgense*), *Gymnocephalus* cernua (Linnaeus, 1758) (syn. *Acerina cernua*) (river Danube; Kakacheva-Avramova et al., 1978); of *Perca fluviatilis* Linnaeus, 1758 (lake Srebarna; Shukerova, 2010; Shukerova et al., 2010); of *P. fluviatilis* (r. Danube; Zaharieva, 2022); 2). *Ac. lucii* of *Silurus glanis* Linnaeus, 1758, Squalius cephalus (Linnaeus, 1758) (rivers Danube, Iskar; Margaritov, 1959); of *Ballerus sapa* (syn. *Abramis sapa*) (Pallas, 1814), *Sq. cephalus*, *Rutilus rutilus* (Linnaeus, 1758), S. glanis, *P. fluviatilis*, Lota *lota* (Linnaeus, 1758), *G. schraetser*, *Bentophilus stellatus* (Sauvage, 1874), *Proterorhinus marmoratus* (Pallas, 1814) (r. Danube; Kakacheva-Avramova et al., 1978); of *P. fluviatilis* (lake Srebarna; Shukerova, 2010); of *L. lota*, *Zingel zingel* (Linnaeus, 1766) (r. Danube; Atanasov, 2012); of *Abramis brama* (Linnaeus, 1758) (lake Srebarna; Chunchukova et al., 2016); of *Alburnus alburnus* (Linnaeus, 1758), *Abr. brama* (r. Danube; Chunchukova, 2017); of *Alb. alburnus* (r. Danube; Chunchukova et al., 2019); of *Sq. cephalus* (r. Osym; Kuzmanova et al., 2019); of *Cyprinus carpio* Linnaeus, 1758; of *Neogobius fluviatilis* (Pallas, 1814), *Babka gymnotrachelus* (Kessler, 1857), *Neogobius melanostomus* (Pallas, 1814) (r. Danube; Zaharieva, 2022); 3). *Allocreadium isoporum* of *Gobio gobio* (Linnaeus, 1758), *Barbus petenyi* Heckel, 1852, *Alburnoides bipunctatus* (Bloch, 1782), *Phoxinus phoxinus* (Linnaeus, 1758), *Sq. cephalus* (rivers Vrabniska, Nishava, Iskrecka, Buchinska, Berkovska; Kakacheva-Avramova, 1969); of *B. petenyi*, *Sq. cephalus* (r. Shiposhnitsa, rez. Iskar; Margaritov, 1977); of *Alb. alburnus* (r. Dunav; Kakacheva et al., 1978); of *B. petenyi*, *Barbus barbus* (Linnaeus, 1758) (Kakacheva-Avramova & Nedeva-Menkova, 1981); of *Sq. cephalus* (r. Osym;

Kuzmanova et al., 2019); of *Alb. alburnus*, *Chondrostoma nasus* (Linnaeus, 1758), *Vimba vimba* (Linnaeus, 1758), *R. rutilus* (r. Danube; Zaharieva, 2022); 4). *Caryophyllaeus laticeps* of *B. barbus*, *B. petenyi* (r. Danube, rez. Iskar, respectively; Margaritov, 1959); of *B. barbus* (r. Danube; Margaritov, 1966); of *B. barbus*, *V. vimba*, *Abr. brama*, *Abr. sapa* (r. Danube; Kakacheva et al., 1978); of *B. barbus*, *V. vimba*, *Abr. brama*, *Ch. nasus*, *Esox lucius* Linnaeus, 1758, *S. glanis* (r. Danube; Atanasov, 2012); of *Sq. cephalus* (r. Osym; Kuzmanova et al., 2019); of *V. vimba*, *Abr. brama* (r. Danube; Zaharieva, 2022); 5). *Contracaecum* sp., larvae of Cyprinus carpio (lakeSrebarna; Shukerova, 2006); of *Aburnus alburnus, Aspius aspius, Abramis brama, P. fluviatilis, R. rutilus* (lake Srebarna; Shukerova, 2010); of *Abr. brama* (Srebarna; Chunchukova et al., 2016); of *Alb. alburnus*, *Abr. brama*, *B. barbus* (r. Danube; Chunchukova, 2017); of *B. barbus* (r. Danube; Chunchukova & Kirin, 2018); of *Alb. alburnus* (r. Danube; Chunchukova et al., 2019); of *R. rutilus* (lake Strebarna; Shukerova, Kirin, 2019); of *Abr. brama*, *Alb. alburnus*, *Ch. nasus*, *V. vimba*, *B. sapa*, *Carassius gibelio* (Bloch, 1782), *C. carpio*, *Leuciscus aspius* (Linnaeus, 1758), *Pelecus cultratus* (Linnaeus, 1758), *Scardinius erythrophthalmus* (Linnaeus, 1758), *N. melanostomus*, *P. fluviatilis*, *S. glanis* (r. Danube; Zaharieva, 2022), etc.

CONCLUSIONS

V. melanosps is a new host recors for *All. isoporum*, *C. laticeps* and *Contracaecum* sp. in Bulgaria. Maritsa river is a new locality of *C. laticeps* and *Contracaecum* sp. All taxa of helminths are autogenous for the Maritsa River, except for *Contracaecum* sp.

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ASSESSMENT OF GROWTH AND MORTALITY PARAMETERS OF *Alosa immaculata* (Bennet, 1835) FROM THE DANUBE DELTA

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Abstract

The aim of this study is to calculate the growth and mortality rates of Alosa immaculata, an important species whose population is in decline. Sampling was realized every month, from March to June 2022 from Sulina Branch Mm 34-18. Each of the specimens that were captured was weighed and measured individually, with weights ranging from 150 to 450 grams and total lengths ranging from 25 to 39 centimeters. The ELEFAN program from FiSAT II was utilized to determine the parameters for the von Bertalanffy growth function, resulting in the values of $L\infty = 36.75$ *cm and* $k = 0.66$ yr^{-1}*. Using the linear regression analysis, the length-weight ratios were predicted based on log-transformed data using the equation* $W = a \times L^b$*. The mathematical relationship between the length and weight for the Pontic shad was:* $W=0.0904 \times Lt^{2.3198}$*. The population of Pontic shad experienced high mortality rates, with a total mortality estimate at 1.83 and a natural mortality rate of 0.87. In addition, the calculated exploitation rate for the Pontic shad population exceeded the optimal value of 0.5, indicating slight overexploitation of the population.*

Key words*: inland fishing, Length-Weight relationship, migratory fish, von Bertalanffy's equation.*

INTRODUCTION

The *Alosa immaculata* is a teleostean, predatory, marine, anadromous fish of the *Clupeidae* euryhaline species family. Its main habitats are the Black Sea, the Sea of Azov and the Caspian Sea (Whitehead, 1985; Coad, 1997). It migrates in large flocks, for reproduction in the rivers (Danube - going up to near the Iron Gates, Dnieper, Dniester, etc.), representing a species with an important economic value for commercial fishing in the Danube Delta, but also in the Danube (Leonov et al., 2022).

The food of the Pontic shad is made up of up to 75% of different species of small marine fish (shrimps, horse mackerels, anchovies, sea urchins) and then of invertebrates, especially crustaceans (Whitehead, 1985).

According to Navodaru & Waldman (2003) and Kottelat & Freyhof (2007), the spawning period of the Pontic shad occurs between April and August, with the main trigger for spawning being water temperatures above 15°C.

Also, the Pontic shad catch was correlated with the waters level of the Danube, especially those in May, reaching the conclusion that the shad catch fluctuations are directly proportional to the water level variations (Smederevac-Lalić et al., 2018).

The migration does not occur over long distances; given that this species only feeds in saltwater marine waters, not freshwater, traveling long distances requires a lot of energy and obviously the cessation of feeding (Balik, 2019).

Smederevac-Lalić et al. (2018) reported that the Pontic shad is highly susceptible to stress, including both natural and human-induced stressors. Due to excessive fishing, anthropogenic impact, extreme climate changes and habitat changes (construction of dams) in the area of the Danube River (Năvodaru, 1996).

The *Alosa immaculata* is included on the IUCN Red List, being considered a vulnerable species, the analysis of growth and mortality parameters analyzed in a study from 2017 (Ibănescu et al., 2017) concluded exactly this aspect. Overfishing can be a major threat to the survival of this species, with unsustainable fishing practices leading to a decline in population numbers.

According to a study carried out in 2016, it was shown that the females of *Alosa immaculata* have a higher survival rate than the male specimens, after reproduction in the Danube River and the Danube Delta. (Tiganov et al, 2016). The same thing was demonstrated in the study published in 2018, including data on shad catches from 2016 when the ratio between the sexes was M/F=0.51 (Năstase et al., 2018).

The primary aim of this investigation is to assess the present status of the *Alosa immaculata* population within the ecosystem of the Danube Delta.

The article presents an analysis of several biological parameters, such as length and weight, alongside the mortality ratio that is crucial in maintaining fish stocks at optimal levels to prevent overexploitation. Overall, the growth and mortality of *A. immaculata* are important factors to consider in the management and conservation of this species. Understanding the growth patterns and mortality rates can help inform effective management strategies to ensure the sustainability of its populations.

MATERIALS AND METHODS

Fishing area. Commercial fishing was realized during the year 2022 in the Danube Delta, Sulina Branch Mm 34-18 (Figure 1).

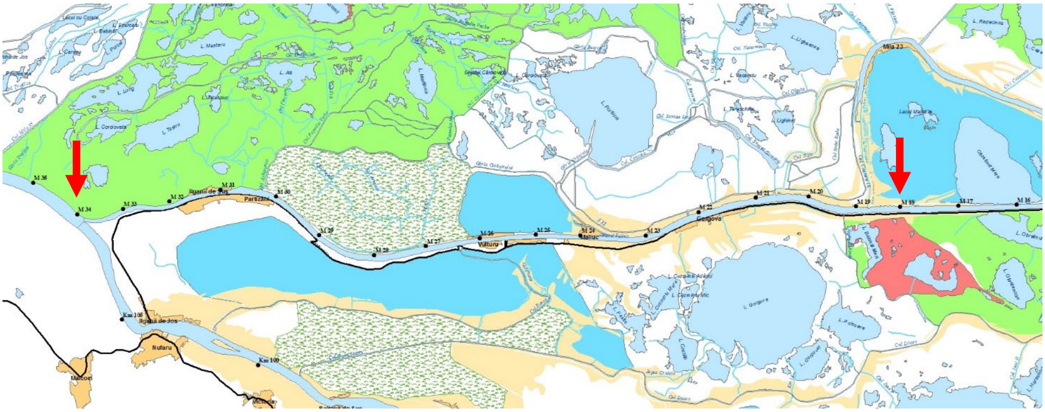

Figure 1. The Sulina Branch Mm 34-18

Data collection. Between March and June, a total of 70 *Alosa immaculata* specimens were captured using gillnets with a mesh size of 30-35 mm. The specimens were collected using a random sampling approach from commercial catches and included representatives from all length classes. The purpose of collecting these specimens was to evaluate the stock of Pontic shad in the studied region.

Total length (TL, in cm), fork length (FL, in cm), and height (h, in cm) were measured with an ichtyometer with an accuracy of 0.1 cm. In addition, the weight (W, in g) of the specimens was determined using an electronic weighing scale with a precision of 0.01 g.

In order to determine the correlations between the length-weight relationship (L-W) for the *Alosa immaculata* population, the relation $W = a \times L^b$ was used, where W represents the weight (in grams) of an individual, and L represents the total length (in cm) of that individual. The growth parameters ($L\infty$, k, t_0) were derived using the length frequency analysis with the ELEFAN model of the FiSAT II program

The mortality rates were calculated using Pauly's methods (1980, 1983), with the total mortality (Z) determined through the length converted catch curve analysis (Ricker, 1975) in FiSAT II. To predict the natural mortality (M), Pauly's formula (1980) was utilized, which takes into account the mean surface temperature (T). The formula was expressed as $\text{Log } M = -0.0066 - 0.279 \times \text{Log } (L\infty) + 0.6543 \times \text{Log } (k) + 0.4634 \times \text{Log } (T)$, where $L\infty$ represents the asymptotic length, T - the average of annual water temperature (12˚C), and k - the growth rate coefficient of Von Bertalanffy.

To calculate fishing mortality (F), the formula F=Z-M (Gulland, 1971) was used, where Z - represents the total mortality, F represents the fishing mortality, and M represents the natural mortality. The formula E=F/Z (Gulland, 1971) was then used to determine the exploitation level (E). Fish stocks were considered easily exploited if the exploitation rate was below 0.5 and heavily exploited if E values were between 0.5-1.

For *data analysis*, the length frequency data were grouped into 3 cm intervals, and the FiSAT II software package (FAO-ICLARM Stock Assessment Tool) and Microsoft Excel 2019 were used.

RESULTS AND DISCUSSIONS

Each generation of Pontic shad, participating in migration, has its own characteristics relating to the size of the body. This is due to the specific environmental conditions in which individuals were born and where they lived, but it is also due to the different responses to these factors (Ibănescu et al., 2017).

From the whole Pontic shad captures, 62.5 % were represented by females and 37.5 % by males. The researchers utilized the growth rings present in the fish scales to determine the age distribution of the population. The analysis identified five distinct age groups, ranging from 2 to 6 years. The highest percentage of individuals captured were three-year-old, representing 33.33% of the total catch, while 26.38% were four-year-old, and 22.22% were two-year-old. The remaining 11.11% were five-year-olds, and a small proportion of only 6.94% were six-year-olds.

The captured fish had total lengths ranging from 25 cm to 39 cm, and there were significant differences between males and females ($p<0.05$). The most frequently observed size category was in the range of 29-31 cm. (Figure 2).

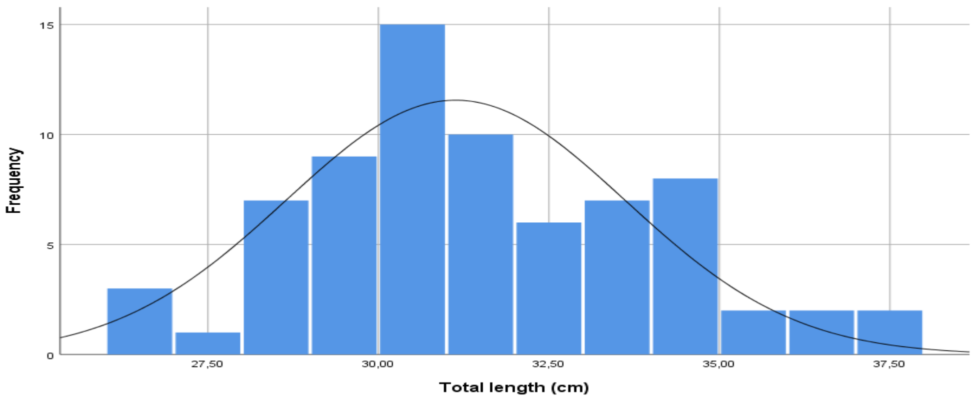

Figure 2. Distribution diagram of length – frequency of *Alosa immaculata*

Table 1 presents the descriptive statistics for the length and body weight of the Pontic shad.

Table 1. Length and body of Pontic shad

Specifications	Length (cm)	Body Mass (g)
Average±SD	31±2.89	274±68.48
Minimum	25	150
Maximum	39	450

The length-weight correlation is a crucial aspect of fishery stock management and ecology (Savaş & Nazmi, 2011). Le Cren (1951) highlighted that studying a fish's diet, reproductive development, and growth can offer valuable information. Tesch (1968) suggested that the coefficient 'b' derived from the L-W correlation can serve as an indicator of the fish's health and the surrounding environment, making it an effective means of comparing fatness and habitats.

The "b" coefficient in this study is 2.3198, indicating a negative allometric growth (increasing of weight is slower than the length). The value of coefficient "b" obtained from the collected samples is lower compared to values reported by Stroe et al. (2020) and Mocanu et al. (2021) in their recent studies on the Danube River. The observed differences may indicate fluctuations in growth, as the length-weight correlation can be affected by various factors such as food availability, water temperature and salinity, or reproduction, as noted by Savaş & Nazmi (2011). Figure 3 presents the growth coefficients "a" and "b" derived from the length-weight relationship, with the population's L-W relationship expressed as $W = 0.0904 \times Lt^{2.3198}$ during the study period. This equation is comparable to the one determined by Iliescu in 1971 ($W = 0.0905 \times Lt^{2.4}$), and a high correlation ($R = 0.895$) was found between length and weight.

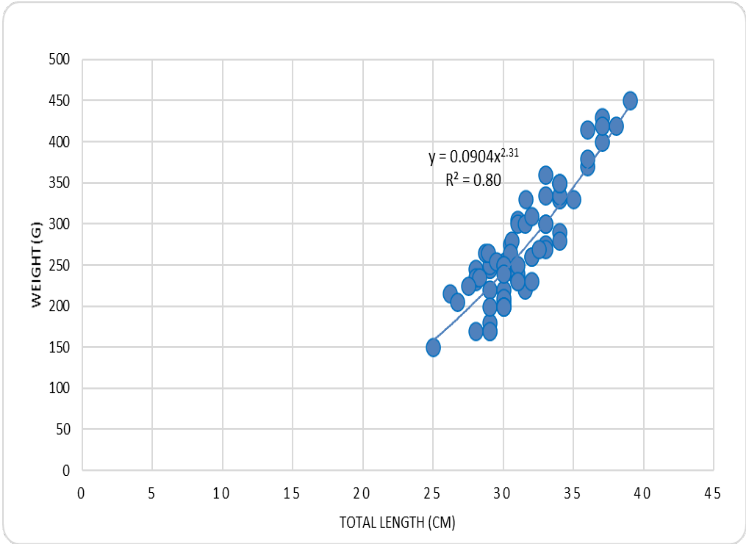

Figure 3. Length-weight correlation (L-W) of *Pontic shad*

The growth indicators $L\infty$, k, and t_0 are constants used in predicting the body size of a fish as it reaches a certain age. In this study, the estimates of these growth variables ($L\infty$, k, and t_0) are shown in Table 2.

Table 2. Growth parameters for Pontic shad in 2022 migration, Sulina

$L\infty$	k	t_0
36.75	0.66	-0.039

In recent studies conducted by other authors, growth parameters have been found to be similar to the data obtained from this study. (Rozdina, 2013; Ţiganov, 2023).

To ensure that fish stocks are not overexploited and remain sustainable, it is crucial to accurately estimate the mortality rates. This is important because if the mortality rate is underestimated, it could result in overfishing, leading to a decline in the fish population. On the other hand, if the mortality rate is overestimated, it could result in unnecessary restrictions on fishing, which could negatively impact the fishing industry. Therefore, accurate estimation of mortality rates is vital for effective fisheries management and the conservation of fish populations.

The estimate of the mortality rates (Z and M) for the Pontic shad population investigated was computed using the FiSAT II computer software package. The values are shown in Table 3 and Figure 4.

Table 3. Mortality rates for Pontic shad, 2022, Sulina

Species	Z	M	F	E
Alosa immaculata	1.83	0.87	0.96	0.53

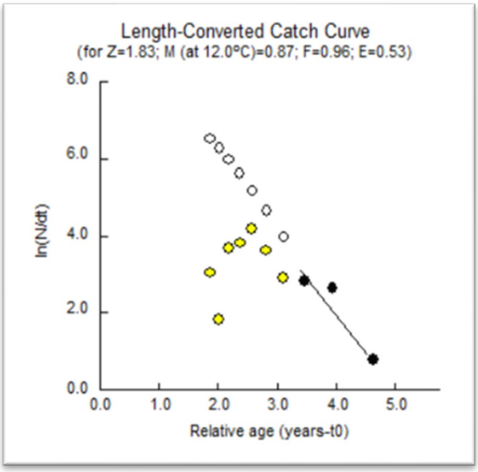

Figure 4. Length converted catch curve of Pontic shad, in Sulina branch, 2022

The exploitation rate (E) of 0.53 for *Alosa immaculata* estimated from the mortality rates, is a little larger than 0.5. It shows that the stock of this species in the Sulina Branch area is slightly overexploited. But it should be remembered that most of the Pontic shad specimens from the Danube River were fished on the St. George arm, which shows that the migration on the other arms of the delta was lower.

CONCLUSIONS

Considering the importance of Pontic shad for commercial fishing in Romania, and the fact that it is considered a vulnerable species and threatened by pollution, overfishing, and climate change, the annual studies lead to a current assessment of stocks that have the role of initiating necessary conservation measures

The study conducted on 70 specimens of *Alosa immaculata* from commercial fishermen caught in the Danube Delta, Sulina Branch Mm 34-18, in 2022 can be concluded:

- The Pontic shad sampled in the studied period had a total length interval between 25 and 39 cm and weight values interval between 150 and 450 g;

- Relationship L–W established was $W=0.0904*Lt^{2.3198}$;

- Growth parameters ($L\infty$, k, t_0) have values similar to those from specialized literature;

- The values of fishing mortality (F) and the rate of exploitation (E) show that *Alosa immaculata* stock is slightly overexploited despite the fact that the vast majority of Pontic shad cohorts migrate to Saint George's arm.

Therefore, it is considered important in future studies to evaluate the stocks of Pontic shad migrating in the Danube River on the Saint Gheorghe branch.

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UPDATED OVERVIEW OF MARINE FISH BIODIVERSITY: SCIENTIFIC SUPPORT FOR AN ECOSYSTEM-BASED MANAGEMENT OF THE DANUBE DELTA BIOSPHERE RESERVE

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Abstract

Fish biodiversity is a key indicator of the health of a waterbody and the structure and function of fish communities are considered good indicators of the ecological status of marine ecosystems. Therefore, the long-term assessment and the development of predictions regarding the size and productive capacity of fish populations are necessary, aiming at ensuring an ecosystem-based management of living resources. In the frame of the revision of the Danube Delta Biosphere Reserve Management Plan, an updated evaluation of fish species present in the marine zone (ROSCI0066) was performed. The scientific fishing (by survey trawling and gillnetting) revealed a rich ichthyofauna: 32 taxa, belonging to 21 families were identified. The species array is diverse, including both economically important fish (turbot, shads, anchovy, sprat) and species of high conservative interest (sturgeons), emphasizing the crucial importance of this area as feeding and spawning ground of Black Sea ichthyofauna.

Key words: *assessment, Danube Delta - Marine Zone, ecosystem-based management, fish biodiversity.*

INTRODUCTION

The Danube Delta is the second largest river delta in Europe. The Danube Delta Biosphere Reserve was created to conserve the area's unique diversity, both biological and cultural, comprising a wide variety of habitats, from wetlands, forests to Marine Protected Areas (MPAs). It has a triple international conservation designation: UNESCO World Heritage Site, Biosphere Reserve since 1990 and Ramsar site due to its importance for migratory birds (Gâștescu, 2021).

At European Union level, the Danube Delta is included in the Natura 2000 network, comprising several sites of Community importance: ROSPA0031 Danube Delta and Razelm - Sinoe Lagoon System, ROSPA0076 Black Sea, ROSCI0065 Danube Delta, and ROSCI0066 Danube Delta - Marine Zone (MEWF, 2016). The latter was the focus of this research, in the frame of the POIM Project "Revision of the Danube Delta Biosphere Reserve Management Plan and Regulation".

An updated overview of marine fish biodiversity in the marine zone of the Danube Delta was absolutely necessary in order to have a sound scientific background substantiating an ecosystem-based management of this MPA, all the more so as information in this area have been scarce and focused mainly on commercial species (Nicolae et al., 2014; Țoțoiu et al., 2018).

Marine biodiversity provides a multitude of valuable ecosystem goods and services and is valued for its direct utility to humans. Despite its important role and contribution to human wellbeing, its reduction has been reported worldwide. The main threats to marine biodiversity include habitat loss, overexploitation, pollution by hazardous substances, eutrophication, and invasions by non-indigenous species (Kappel, 2005; Venter et al., 2006). Restoring marine biodiversity through sustainable fisheries management, pollution control, maintenance of essential habitats, and the creation and better management of marine reserves are some of the opportunities for investments that can support the productivity and reliability of goods and services that the ocean provides to humanity (Palumbi et al., 2009).

Biodiversity includes four main components: (I) genetic diversity - which refers to the genetic variation that occurs among members of the same species; (II) species diversity -

which refers to the variety of species or other taxonomic groups in an ecosystem); (III) ecosystem diversity - which refers to the variety of biological communities found on earth; and (IV) functional diversity - which refers to the variety of biological processes, functions or characteristics of a particular ecosystem (Thorne-Miller, 1999). Species diversity is the basis for the diversity of higher taxa and ecological associations such as communities and biomes (Kiester, 2013).

The main objective applied in modern fisheries management is to maintain the diversity of species (II) within the marine ecosystem, since marine organisms contribute to many critical processes that have direct and indirect effects on the health of the oceans and humans (Steele et al., 2001).

The most widely used metric of biodiversity is species richness (Appeltans et al., 2012). Species richness is a fundamental property of any biotic assemblage (Foggo et al., 2003). Accurate estimates of richness, based on the presence or absence of species, can be performed; in turn, reliable correlations of abundance/dominance may be an addition to the potential value of such techniques in biodiversity inventory (Figure 1) (Simberloff & Moore, 1997).

Also, within the EU Marine Strategy Framework Directive (MSFD) the conservation status of vulnerable fish species has been selected as an appropriate measure to report information on the biodiversity of the marine environment, especially regarding the impact of fishing on diversity (Borges et al., 2010). The MSFD puts biodiversity in the center of the assessment of marine environmental status. The descriptor (D1) on biodiversity has the following target to contribute to the achievement of the Good Environmental Status (GES): "Biological diversity is maintained. The quality and occurrence of habitats and the distribution and abundance of species are in line with prevailing physiographic, geographic, and climatic conditions" (Borja et al., 2010).

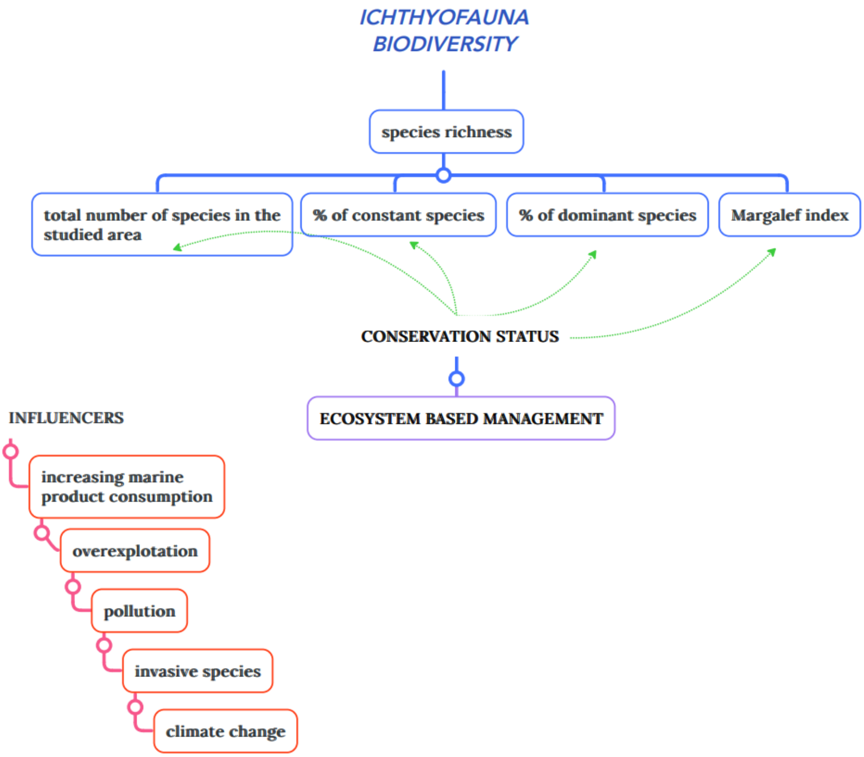

Figure 1. Ichthyofauna biodiversity scheme: components and influencers (*Original figure*)

Thus, the study of the ichthyofauna biodiversity is highly important and in recent years an increase in the number of marine species identified on the Romanian coast has been observed. In the last 10 years, 71 species of fish have been identified, of which 14.28% are rare species, 28.57% are dominant species and 57.15% are common species (Niță et al., 2022).

MATERIALS AND METHODS

In the frame of the revision of the Danube Delta Biosphere Reserve Management Plan, an updated evaluation of fish species present in the marine zone (ROSCI0066) was performed.

Scientific fishing

The inventory activity of marine fish species was carried out by organizing 6 scientific fishing expeditions, between October 2019 and September 2021.

The methodology and techniques that were used both for data collection, verification, processing and analysis, as well as for the assessment of fish biodiversity are those used in the Black Sea basin and in accordance with the most up-to-date international methodology (Carpentieri et al., 2020).

The scientific survey for the inventory of fish species was conducted with:

▪ the "Steaua de Mare" research vessel, equipped with specialized fishing gears: demersal and pelagic trawls (Figure 2).

Figure 2. Trawling activity (Original photo)

The trawl is a truncated cone-shaped fishing gear, equipped with its own arming system, towed with the help of a vessel by means of connecting elements (sweeps, wing-lines, bridles, trawl doors etc.). It can be towed either close to the seabed, targeting demersal species, or in the mid-water, targeting pelagic fish schools (Țoțoiu et al., 2017).

▪ motor-equipped pneumatic boat for gillnet survey fishing (Figure 3).

The gillnet is a net-type fishing gear consisting of a single net wall with a vertical operating position generated by reinforcements provided at the top (floats) and bottom (leads). Gillnetting is a fishing method by entangling and hooking, which consists in blocking the direction of movement of the fish with a vertical wall of netting, in the meshes of which the fish remains hooked and entangled when it tries to pass (Anton, 2016).

Figure 3. Gillnet fishing activity (Original photo)

During the scientific expeditions, observations were made regarding the marine species sampled during 85 trawling operations with the trawl and additional information was obtained from 12 stations where samples were taken with gillnets; nets of different mesh sizes were used, so that all types of habitats and depths were covered, and fish samples were analyzed both on board, but also in the laboratory.

Determination of fish species

For the systematic identification and classification of the collected individuals, the "*Determinator of the Main Fish Species of the Black Sea*" (Radu & Radu, 2008), as well as the updated fish biodiversity atlas (Niță et al., 2022) were used (Figure 4).

Length data

The total length was analyzed with the ichthyometer. Within a sample, the individuals were assigned to length classes at a range of 50 mm (Figure 4b).

Weight data

The total weight of the individual was measured in grams using an electronic scale (Figure 4a).

Figure 4. Sample sorting onboard: a - fish length classes; b - weight measurement (Original photos)

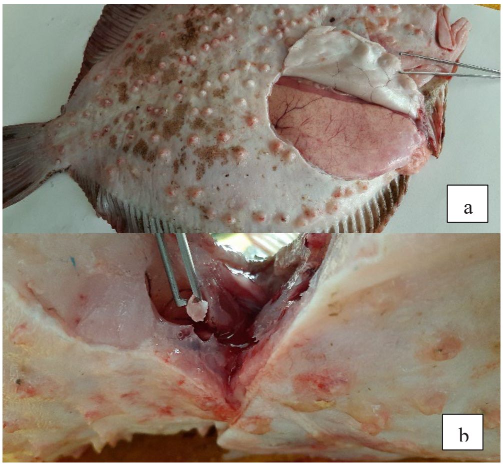

Figure 5. The study of individuals in the laboratory: a - gonad analysis; b - age reading (Original photos)

Gender identification data

To identify the sex, an abdominal incision was made, then the gonads were collected to be subsequently examined according to superficial vascularization, color, transparency, consistency and volume (Figure 5a).

Age reading

Age reading was performed by extracting the otoliths and subsequently analyzing them under a binocular microscope (Figure 5b).

Biodiversity indices

To estimate the species richness within the ichthyofauna, in the studied area, the Margalef Index was calculated (Magurran, 2004):

$$DMg = (S-1)/\ln N$$

where *S* is the number of species recorded in one sample and *N* is the total number of individuals (from all species).

It is considered that values <2 represent a low species diversity in the analyzed community and values <5 indicate a high species diversity (Magurran, 2004).

Also, other ecological indicators were analyzed: constancy and dominance (Gomoiu & Skolka, 2001).

Constancy (frequency) is an indicator that expresses the continuity of a species in a certain territory. It is a structural type of indicator, representing the percentage ratio between the number of samples in which a certain species appears and the number of analyzed samples. Depending on the value of constancy in the samples, the species can be divided into the following categories: C1 - accidental species, present in 1-25% of the samples; C2 - accessory species, present in 25.1-50% of the samples; C3 - constant species, present in more than 50.1% of the samples (Gomoiu & Skolka, 2001).

Dominance expresses the so-called relative abundance of a species, representing the ratio between the numbers of a certain species and the sum of the numbers of the other species in the studied area (Gomoiu & Skolka, 2001). Depending on the percentage value, the species are divided according to dominance into: D1 - underrepresented species, when the percentage is below 1.1%; D2 - recessive species, when the percentage is between 1.2 - 2%; D3 - subdominant species, when the percentage is between 2.1 - 5%; D4 -dominant species, when the percentage is between 5.1 - 10%; D5 - eudominant species, when the percentage is > 10.1% (Gomoiu & Skolka, 2001).

RESULTS AND DISCUSSIONS

Ichthyofauna represents a basic component of marine biodiversity, and, according to bibliographic sources, the fish fauna of the Black Sea includes over 140 species and subspecies (Radu et al., 2008). Out of the 140 species of fish reported in the Black Sea, in the catches from the Romanian coast, as well as in the research expeditions carried out by the experts of NIMRD "Grigore Antipa" Constanța in the last decade 68 species of fish were identified (Niţă et al., 2022).

Following the scientific fishing expeditions carried out in the marine zone of the Danube Delta (ROSCI0066) in the frame of the Management Plan revision project, 32 fish species were identified, belonging to 21 families, of which the three species of sturgeons (*Acipenseridae*) are of particular conservation importance (Figure 6).

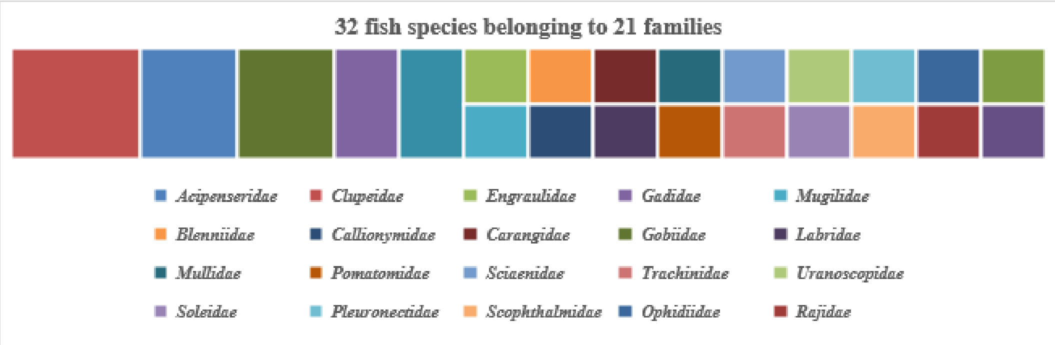

Figure 6. Treemap of fish species identified in the Danube Delta - Marine Zone during the study (2019-2021)

The state of conservation of plants and animals is one of the most used indicators for evaluating the state of ecosystems and their biodiversity. On a global scale, the primary source of information on the conservation status of plants and animals is the International Union for Conservation of Nature (IUCN) Red List of Species. This list is a powerful tool to inform and catalyze action to conserve biodiversity and change the policies essential to protecting the natural resources we need to survive. It also provides information on range, population size, habitat and ecology, use, trade, threats and conservation actions that will assist in making the necessary sustainable management decisions (IUCN, 2023).

Regarding the conservation status of the identified species, the 3 species of *Acipenseridae* are classified as Critically Endangered (CR). Another 5 species fall into the Vulnerable (VU) category and another 3 in the Data Deficient (DD) category (Table 1). All other fish species identified in the marine area of the DDBR are Least Concern (LC). Two other species of Community interest were also identified in the study area, one being included in the list of vulnerable species: *Alosa immaculata* (Table 2).

Table 1. Conservation status of the fish species identified in the marine zone of the Danube Delta

<table>
<tr><th colspan="5">CR</th></tr>
<tr><td>Huso huso</td><td colspan="2">Acipenser gueldenstaedtii</td><td colspan="2">Acipenser stellatus</td></tr>
<tr><th colspan="5">VU</th></tr>
<tr><td>Alosa immaculata</td><td>Pomatomus saltatrix</td><td>Umbrina cirrosa</td><td>Squalus acanthias</td><td>Dasyatis pastinaca</td></tr>
<tr><th colspan="5">DD</th></tr>
<tr><td>Ophidion rochei</td><td colspan="2">Hippocampus guttulatus</td><td colspan="2">Nerophis ophidion</td></tr>
</table>

Table 2. Species of Community interest identified in the study area

Specification	*Huso huso* (Linnaeus, 1758), Genus *Huso*, Beluga
N2000 Code	2489
	Acipenser gueldenstaedtii (Brandt & Ratzeburg, 1833), Genus *Acipenser,* Russian sturgeon
N2000 Code	5040
	Acipenser stellatus (Pallas, 1771), Genus *Acipenser*, starry sturgeon
N2000 Code	2488
	Alosa immaculata (Bennett, 1835), Genus *Alosa*, Pontic shad
N2000 Code	4125
	Alosa tanaica (Grimm, 1901), Genus *Alosa,* Azov shad
N2000 Code	4127

Regarding the application and analysis of biodiversity indicators for the study period, we used all the information collected during the scientific expeditions.

In the autumn expedition of 2019, observations were made in 20 stations and the values of Margalef Index (DMg) varied between 0.47 to 3.47 (Figure 7).

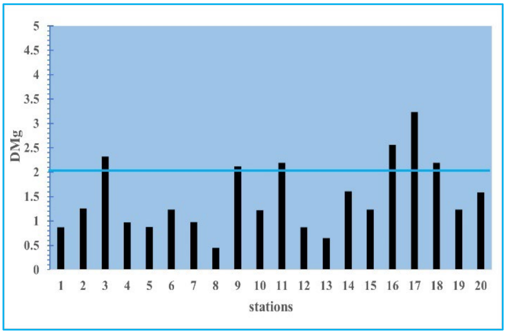

Figure 7. Margalef Index values in the 2019 survey

It is also observed that in several stations the values have exceeded the threshold value of 2, considered to indicate a moderate diversity of species in the study area.

Regarding the other indicators, the following results were obtained:

- whiting, sprat, Azov shad and piked dogfish = constant species (C3);
- gobies, horse mackerel and starry sturgeon = accessory species (C2);
- the other 11 species = accidental species (C1) (Figure 8).

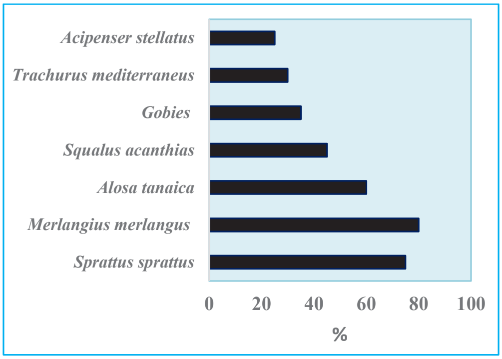

Figure 8. The representation of species (%) consistently identified in the samples during the 2019 survey

During the summer expedition of 2020, we analyzed samples from 13 stations. Thus, it was observed that the value of the Margalef index exceeded the threshold value in several stations, which indicates an increased diversity of species (Figure 9).

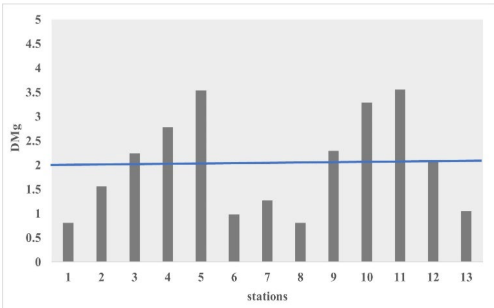

Figure 9. Margalef Index values in the summer 2020 survey

Regarding the constant presence of the species identified in the samples, it can be observed that whiting (*Merlangius merlangus)* had the highest value (Figure 10).

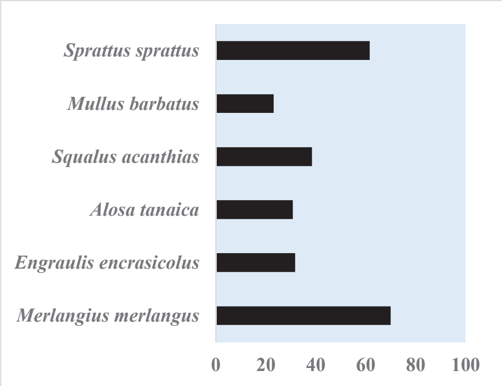

Figure 10. Representation of the constant species in the analyzed samples (summer 2020)

Two of the identified species had a high percentage of constant presence in the samples, the others registering lower values (namely sprat and whiting).

During the autumn expedition in 2020, samples from 18 stations were analyzed. The Margalef indicator had the highest value of 3.58 in one of the sampling stations (Figure 11).

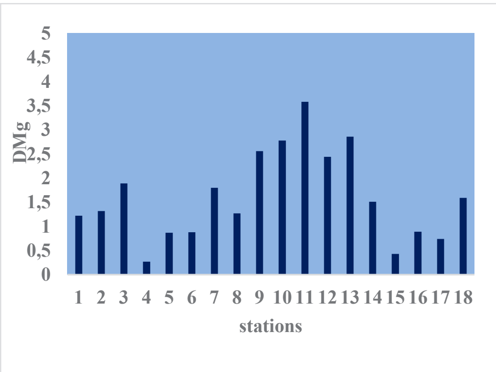

Figure 11. Margalef Index values in the autumn 2020 fishing survey

Analyzing the presence of species, it was observed that the species with the most constant presence was the horse mackerel (*Trachurus mediterraneus)* in more than 30% of stations (Figure 12).

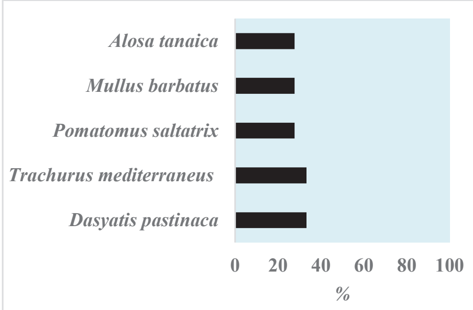

Figure 12. The graphic distribution of the constant species in the analyzed samples (autumn 2020)

Regarding the spring expedition in 2021, we analyzed samples from 10 stations. The Margalef indicator had values above the threshold value in approximately half of the analyzed stations (Figure 13).

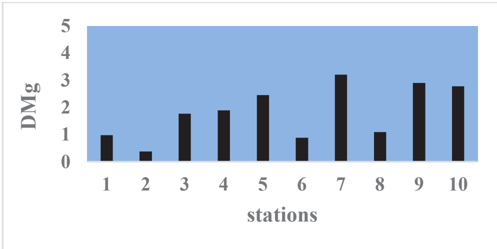

Figure 13. Margalef Index values in the spring 2021 survey

Regarding the species presence in samples, whiting was present in all the stations (Figure 14).

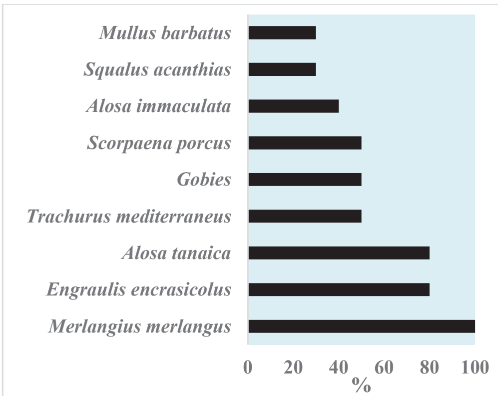

Figure 14. Representation of the constant species in the analyzed samples (spring 2021)

Also, other two species registered a very good presence in the samples (80%): anchovy (*Engraulis encrasicolus*) and Azov shad (*Alosa tanaica*) (Figure 14).
During the summer survey of 2021, observations were made from 16 stations (Figure 15).

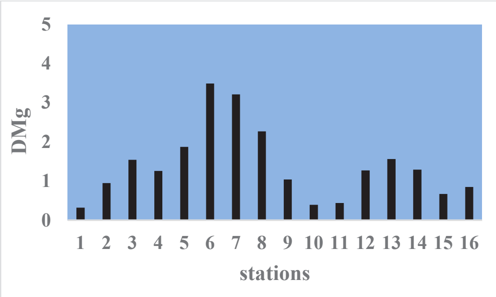

Figure 15. Margalef Index values in the summer 2021 survey

In this expedition the diversity indicator registered values which show a high diversity of fish species in the analyzed area. 22 species of fish were identified during the survey.
Regarding the presence in the analyzed samples, the sprat (*Sprattus sprattus*) was the most constant species (Figure 16).

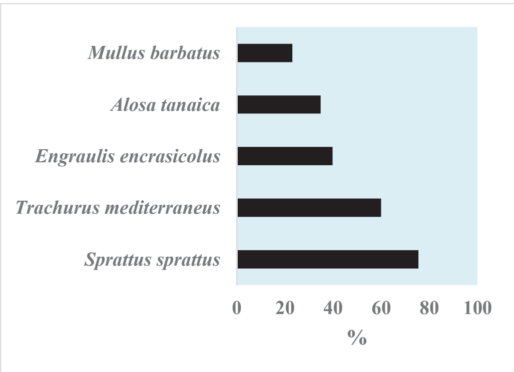

Figure 16. Representation of the constant species in the analyzed samples (summer 2021)

Regarding the autumn expedition in 2021, observations concerning the fish species diversity, were made from 8 stations (Figure 17).

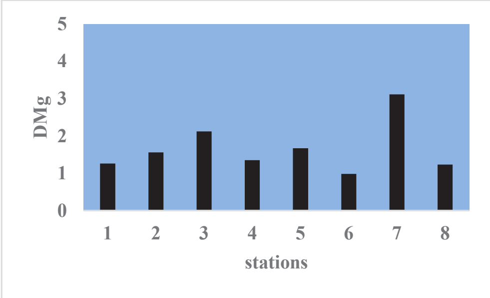

Figure 17. Margalef Index values in autumn 2021 expedition

During this expedition, lower values of the diversity indicator were observed.
Referring to species presence in the analyzed samples, it was observed that sprat and horse mackerel consistently prevailed (Figure 18).

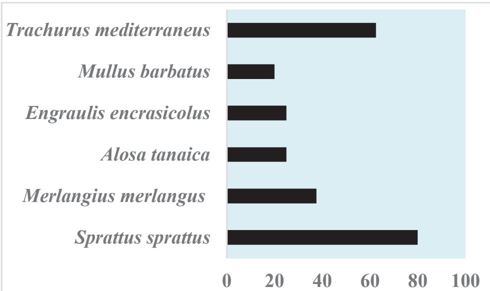

Figure 18. The graphic distribution of the constant species in the analyzed samples (autumn 2021)

As regards the dominance indicator, for the entire study period, the presence of 5 species in the eudominant category and 3 species in the dominant category was highlighted, the rest of the species belonging to the other categories (Table 3).
Among the eudominant species the prevalence of small commercial interest species is to be remarked.
Moreover, the dominance of the species in the samples varied depending on the season in which the scientific expedition was carried out.
In the spring fishing season, sprat and Azov shad predominated in higher percentages, while in the summer season the percentage of anchovies increased, and in the winter season, horse mackerel was dominant.

Table 3. List of species according to the category of dominance indicator

D1 - underrepresented (<1.1%)	D2 - recessive (1.2-2%)	D3 - subdominant (2.1-5%)	D4 - dominant (5.1-10%)	D5 - eudominant (> 10.1%)
Huso huso ***Acipenser gueldenstaedtii*** ***Gaidropsarus mediterraneus*** ***Chelon auratus*** ***Parablennius tentacularis*** ***Callionymus pusillus*** ***Aphia minuta*** ***Umbrina cirrosa*** ***Uranoscopus scaber*** ***Ophidion rochei*** ***Hippocampus guttulatus*** ***Nerophis ophidion***	*Clupeonella cultriventris* *Gobius niger* *Symphodus rostratus* *Trachinus draco*	*Neogobius melanostomus* *Pegusa lascaris* *Platichthys flesus* *Scophthalmus maeoticus* *Dasyatis pastinaca* *Scorpaena porcus* *Acipenser stellatus*	*Alosa immaculata* *Squalus acanthias* *Pomatomus saltatrix* *Mullus barbatus*	*Sprattus sprattus* *Merlangius merlangus* *Engraulis encrasicolus* *Alosa tanaica* *Trachurus mediterraneus*

Two of the sturgeon species (beluga and Russian sturgeon) were underrepresented, while starry sturgeon was subdominant. Other subdominant species include turbot, stingray and gobies, which are also important for commercial fisheries. The rare species (E.g. *Symphodus rostratus, Trachinus draco, Umbrina cirrosa, Uranoscopus scaber, Ophidion rochei, Hippocampus guttulatus* etc.) were either recessive or underrepresented, yet their presence in the catches accounts for a rich biodiversity of the investigated area.

Scientific support for an ecosystem-based management of the Danube Delta - Marine Zone

The information collected during the study regarding the species richness, indicators of diversity, the constant presence of some species in certain areas and periods, are the basis for the initialization of an ecosystem-based management of the Danube Delta Biosphere Reserve, in the frame of the revised Management Plant, which is currently under official approval.

Ecosystem-based management (EBM) is place-based, considers connections within and among ecosystems (including a balanced and integrated view of social and natural components), and focuses on maintaining the long-term ability of ecosystems to deliver a range of services (Grumbine, 1994). A shift to EBM requires management actions across a range of spatial scales and attention to connections among spatial as well as governance units. The revision of the Danube Delta Management Plan is an optimal occasion to include specific conservation measures aiming at fostering the fish biodiversity of the area.

Given the high richness of ichthyofauna in the analyzed area (32 species), as well as the presence here of vulnerable and critically endangered species, it is aboslutely necessary to apply specific measures aiming to maintain the balance within the Danube Delta - Marine Zone (ROSCI0066).

Whereas the major themes covered by the **revised Management Plan** are biodiversity conservation and management (species and habitats of conservative interest) and detailed biodiversity inventorying and monitoring, the measures proposed focus in two directions: conservation activities and prohibitive measures.

Fish conservation activities include: enforcement of stricter fishing regulatory measures in ROSCI0066 (mesh size, number of gears, thread fineness etc.); ensuring compliance with the turbot fishing prohobition period and the provisions regarding permitted gear and the minimum size of retained specimens; permanent monitoring and control of the commercial fishing activity in ROSCI0066 in order to ensure the accuracy of data from the fishery for the correct estimation and management of stocks; facilitation and implementation of “ghost net“ fishing practices; promotion and stimulation (including financial) of mollusk fishing and harvesting using low-impact gears; inventory and monitoring of conservative interest fish species.

Restrictive measures, on the other hand, include: total ban on fishing for the three species of sturgeons throughout the year (except for fishing for scientific purposes and, in this case, with their immediate release in a viable state); prohibition of commercial fishing for Pontic shad (*A. immaculata*) throughout the year in front of the mouths of the Danube (in accordance with the annual prohibition orders); prohibition of the use of the beam trawl and the classic hydraulic dredge in the territory of ROSCI0066 below the 20 m isobath; between the 20 - 40 m isobaths, their use will be allowed only by alternating in time and space the perimeters subject to the impact with perimeters of biological recovery, following the completion of specialized studies; only small low-impact hydraulic or hand-operated dredges are proposed to be allowed (Niță et al., 2021); ban on the use of the pelagic trawl in ROSCI0066 below the 20 m isobath; ban of the deliberate introduction into ROSCI0066 of invasive species.

All proposed measures are in line with the applicable fisheries legislation in Romania and were openly debated with stakeholders within a participatory approach process.

CONCLUSIONS

During the analyzed period, 32 fish species were identified as a follow-up of scientific fishing. Regarding the conservation status of the identified species, the three species of *Acipenseridae* are classified as Critically Endangered (CR), another five species fall into the Vulnerable (VU) category and another three in the Data Deficient (DD) category.

Biodiversity indices were calculated and, as respects of Margalef Index values, these ranged from 0.32 - 3.58, indicating a high fish diversity in certain stations. Regarding the information obtained in the study concerning the presence of some species in the samples, the constant presence of some species of commercial interest was highlighted (sprat, horse mackerel, anchovy). However, a constant presence in the samples of some vulnerable (dogfish) or threatened species (starry sturgeon) was also observed.

Referring to the dominance indicator, only five species were eudominant (sprat, whiting, anchovy, Azov shad, horse mackerel and red mullet) and three species were dominant (Pontic shad, dogfish and bluefish). Other species were identified in lower percentages, yet the fish fauna array of the Danube Delta - Marine Zone indicates a high diversity.

All the information acquired regarding fish species richness, indicators of constant presence and dominance are the scientific background for the newly revised Management Plan of the Danube Delta Biosphere Reserve, which contains the proposed targeted measures (both conservative and restrictive) aiming at ensuring an ecosystem-based management of marine fish resources in the area.

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MAIN CAUSE FOR ADMITTANCE OF WHITE STORKS IN WILDLIFE REHABILITATION AND BREEDING CENTRE IN BULGARIA

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Abstract

The White Stork (Ciconia ciconia) is one of the only two taxa representatives of the Family Ciconiidae (Storks) that nest in Bulgaria. The species is protected both on the territory of the country and the EU, and is included in the subject and conservation objectives of many SPAs in the country, part of the European ecological network NATURA 2000. In this paper we present the number, etiology, condition and treatment outcome of over 2,900 specimens of the species accepted for treatment in the period 1999 - 2021. These are patients of the Green Balkans Wildlife Rescue Center in Stara Zagora, which is leading unit for ex-situ conservation of wildlife protected and rare bird species not only in the country but also on the Balkan Peninsula. The outcome of the treatment of all white storks is presented in 4 main categories – returned to nature, housed for aviary breeding, redirected to other ex-situ structurers, as well as lethal outcome. We evaluated the influence of different etiological factors, age and season on the treatment outcome.

Key words: *ex-situ conservation, protected and rare birds, wildlife*

INTRODUCTION

In the early 2000s, the European White Stork (*Ciconia ciconia* Linnaeus, 1758) breeding population was estimated at between 180,000–220,000 breeding pairs (BirdLife International, 2004). White storks from western Europe migrate through Iberia and via the Strait of Gibraltar to get to their winter fields either in flood plains of large rivers south of the Sahara desert in Western Africa or on the Iberian peninsula (Tortosa et al., 1995). Its international protected status is Least Concern (BirdLife International, 2012). In Bulgaria the breeding population counted 4818 pairs in 2004, which was raised by 14.7% compared to 1994, but it is still listed as a “vulnerable” bird in the Bulgarian Red Data Book (Golemanski, 2011). Through Bulgaria passes one of the busiest migrating routes of the White Stork, which is its way to Africa and back to Europe. Nearly 80 percent of the white stork world population passes through the country (Gerdzikov et al., 2014). White Storks usually build their vast observable nests near to human settlements (Daniluk et al., 2006). White Stork more often choose various agricultural areas, villages, settlements, and even suburbs of larger towns or abandoned and active farms in which they build their nests. Single trees and tree groups, power lines, and water towers in agricultural landscapes were examined in detail as common White Stork nest sites (Vaitkuviene & Dagys, 2015). In our country, a huge part of their nests are located on risky facilities such as electric poles, old roofs, chimneys, various buildings and even churches (Golemanski, 2011; Cheshmedzhiev et al., 2016). So far, the white stork is one of the best known and closest to people birds in Europe and is an object of interest to children (Schüz, 1959).

White Stork is considered as a typical species of the present agricultural landscape and it is a good indicator of sustainable and eco-friendly agriculture (Kosicki & Indykiewicz, 2011). The diet of the White Storks as an opportunistic carnivore is well studied and it includes variety of small vertebrates (such as mammals and amphibians) and larger invertebrates (insects and earthworms) in predominantly open areas and wetlands (Pinowska & Pinowski, 1985). In Bulgaria, it observes the same ecological niches, expressed mainly in visiting pastures and wetlands and uses insects for its main food - mainly grasshoppers and beetles. Typical aquatic inhabitants are an exception in the diet. The white stork's primary way of foraging is by roaming mesophytic grasslands (abandoned arable fields and overgrown grasslands with low, moderately humid, vegetation) and locating its prey (Milchev et al., 2013). Therefore, essential for the existence and reproductive success of the White Stork is the presence of grazing animals, a high number of insect populations, mainly large dung beetles, a reduction in the use of chemical agents in agriculture (fertilisers and pesticides) and the presence of small wetlands (Janiszewski et al., 2013). Birds are increasingly settling on overhead power line poles, and the share of tree-nesting pairs is gradually decreasing. It should be noted that in recent years, overhead power poles have increased dramatically, leading to an increase in nesting on power poles. This is most likely a consequence of a gradual change in nesting behaviour of the white stork due to the growing population, on the other hand there is a lack of traditional nesting sites (on trees and on building roofs) that are either already occupied by birds, or are not suitable due to various factors (e.g. poor maintenance) (Vaitkuviene & Dagys, 2015). The inaccessibility of such nests compared to those built on trees and roofs provides them with protection from predatory mammals, therefore increasing the breeding success of white storks (Tryjanowski et al., 2009). On the other hand, these nests are unstable and dangerous, due to their close proximity to wires, they pose serious threats to breeding birds and their young (Kaługa et al., 2011). Other factors, such as a change in the range of the species and the environmental climatic conditions of the wintering grounds, probably contribute to a change in the population size of the white stork (Schaub et al., 2005). Due to the closeness of the White Stork's habitat range to humans, they are among the bird species that often suffer from accidents caused by anthropogenic factors, but are also the most likely to be provided with care and protection in the event of accidents, injuries and disabilities. Today's reports of injured storks make up the largest proportion of all interventions reported to wildlife NGOs. The main objective of the present study is to identify the leading causes of accidents and admissions of white storks to a rehabilitation centre.

MATERIALS AND METHODS

A retrospective analysis was made of the white storks admitted during the period 1999-2020 to the Wildlife Rehabilitation and Breeding Centre (WRBC) Green Balkans - Stara Zagora, using the database for recording original medical records, through authorised personal access.

When analysing the database of the WRBC Green Balkans - Stara Zagora, we divided the patients according to the reasons for admission, forming two main groups - natural causes and anthropogenic causes leading to wildlife incidents. We separated the two main groups of causes into subcategories, according to the established etiological factors of a native character (for natural causes) and the result of human actions (for anthropogenic causes). Categories and subcategories of reasons for admission were classified based on data from history (location information and information about people who reported the animal in distress), initial diagnosis (injuries, specific signs, clinical examinations) and etiology of the harmful factor (Molina-Lopez et al., 2011). The subcategories of anthropogenic causes were grouped as follows - Electrocution and collision with power line, Confiscated, Gunshot, Collision with vehicles and Other anthropogenic factors. We divided subcategories to natural causes as - Fallen from a nest, Extreme weather, Infection disease, Rivalry.

With regard to the environmental protection status of the patients of the WRBC Green Balkans - Stara Zagora, it is necessary to clarify that all species of wild fauna, including protected, rare and vulnerable, fall under the management of the Ministry of Environment and Water (MOEW), which is the competent authority in the Republic of Bulgaria for the protection of wildlife. The MOEW and the regional subdivisions - Regional Inspectorate of Environment and Water (RIEW), which at the local level take care of the wildlife's protection, including receiving and referring for therapy of discovered injured or distressed specimens, directly apply the provisions of the Biological Diversity Act (BDA). In addition, patients are also referred to WRBC Green Balkans - Stara Zagora for treatment by other organisations and private entities - private persons or companies, non-governmental organisations, zoos, private veterinary clinics and private veterinarians, municipal employees, as well as employees of the Ministry of Agriculture, Food and Forestry (MAFF), national or natural parks, etc.

For the purpose of tracking the trends in the change of the number of patients and the reasons for their admission, we analysed the frequency distribution of patients in the period from 1995 to 2019, as well as their fluctuations during the different months of the year.

Variations in the number of incidents were investigated for different age groups: Egg - when, for various reasons, the eggs were taken from the nest and subsequently placed in an incubator and possibly reared in the WRBC; Juvenile - birds with only down plumage or with initial growing feathers, as well as birds with grown plumage with narrow feathers and a black beak, before making their first flight; Immaturus - change of feathers has begun - narrower (characteristic of a young bird) and wider (characteristic of an adult bird), light-red beak with a dark tip and plumage characteristic of an adult bird (broad feathers) are found; Adultus – fully broad feathers and dark red beak (Svensson et al., 2009).

After accepting a live bird for treatment, four different outcomes can follow: 1) Euthanasia, which is humanely assisted death applied to animals with a poor prognosis and whose continued existence is accompanied by pain and suffering that cannot be alleviated; 2) Lethal outcome in animals for which treatment fails and complications occur leading to death; 3) Release into the wild, of successfully cured individuals with good prospects for adaptation in the wild, and 4) Captivity, for animals that cannot be released and have been kept permanently in captivity due to their poor prognosis for survival in the wild. Such animals are directed to zoos or rescue centres and/or are included in breeding programs. Regarding the birds that were found dead and sent to the WRBC for all of them a diagnosis after examination and cause of death were determined.

The data were processed with IBM SPSS Statistics (SPSS-Inc., 2019, SPSS Reference Guide 26 SPSS, Chicago, USA) using descriptive statistics with frequency distribution tables. The correlation between different variables was investigated with the Pearson correlation coefficient. All categorical data were organised in 2×2 cross-tables.

RESULTS AND DISCUSSIONS

Data was analysed for 2,963 White Storks (*Ciconia ciconia*) that passed through the WRBC. The main reason for admission of the species to the rescue centre is of a native character (48.8%), followed by unknown reasons for 42.8%, with the smallest share of 8.3% for anthropogenic factors (fig. 1).

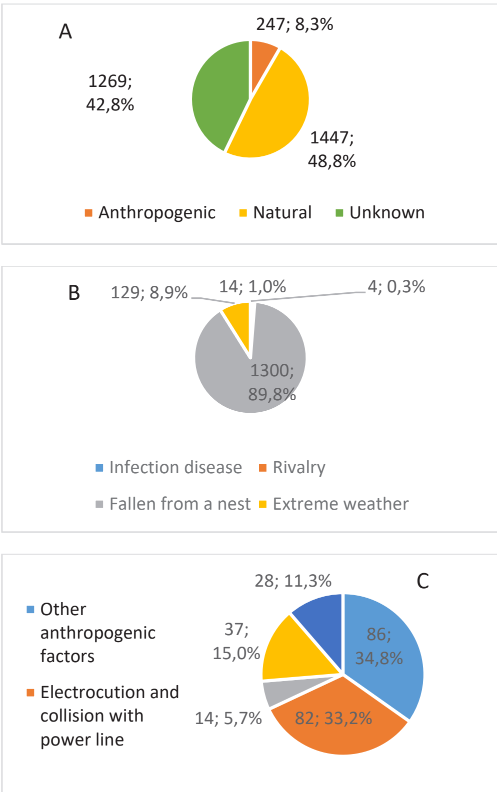

Figure 1. Primary causes for admission:
A) Proportion of anthropogenic and natural causes;
B) Etiological factors of a native character;
C) Etiological factors of anthropogenic character

Due to natural causes, accidents most often occur with young birds when the chances of survival of young birds is lower (Kanyamibwa et al., 1993). This statement coincides with the findings of other studies, which showed that species living in urban and surrounding areas are most often admitted to rescue centres and most of them are juveniles presumed to be abandoned or orphaned (Wimberger & Downs, 2010). The White Stork *Ciconia ciconia* can vary the period of its migration in response to current weather conditions on breeding grounds, but cannot respond to extreme weather events. Due to the increasing frequency of extreme weather events caused by climate change, an occasional accident or a natural disaster, most often associated with sudden temperature changes (extreme weather) are the second most common cause of a natural cause for incidents of the species (8.9%, n=129) (Tobolka et al., 2015). Infectious diseases in the species are not a common reason for admission, they present only 0.3% of natural causes and here it refers mostly to viral diseases, such as Avian influenza and West Nile fever. A small percentage (1.0%) of birds being injured and needing treatment due to intraspecies strife, mostly during breeding and searching for nesting territories, as well as interspecies aggression, is reported. The high mortality rate among storks also appears to be a result of their behaviour, such as using electric poles as a place to rest and to avoid the possibility of encounters with terrestrial predators. The large number of birds looking for a resting place inaccessible to terrestrial predators can cause interspecies conflicts, due to the limited space on the poles, this in turn predisposes to collision with power lines (Demerdzhiev, 2014). When analysing the cause of bird trauma, the results show that 42.8% of the cases remain of unclear etiology (lack of information about the circumstances of the trauma or accident). This high percentage coincides with the ratio of total number of admitted patients of WRBC with the same etiology from our previous study (Lazarova, 2022), as well as in other centres in Europe (Molina-Lopez et al., 2017). Despite the relatively low percentage of anthropogenic factors, leading to wild bird casualties, we consider them in detail because of the impact of human activity on the species. In this category, the leading share in accidents is caused by electrocution and collision with power lines - 33.2%. Power lines are one of the main sources of human-caused mortality in birds due to electrocution or collision, but many species use power poles as a nesting structure. The white stork increasingly nests on poles located near landfills, surrounded by a large proportion of grassland and when close to sources of freshwater (water body or river) and other occupied pylons, further greatly increasing the electrocution rate of individuals (Burdett et al., 2022). One of the most common reasons for admission of protected species to rehabilitation centres is their illegal ownership by private individuals (Molina-Lopez et al., 2017). Despite legislation on protected species in Europe (Nikolova, 2010), the illegal capture and retention of wild birds is still a serious problem, having a negative impact, especially on songbirds (BirdLife International, 2011) and reptiles (Perez et al., 2004). Unlike other countries, where the capture of stork species is widespread (Singh & Chanratanak, 2012), in Bulgaria, although these birds are not used for entertainment, meals, etc., due to the fact that the species habitat is close to people it makes the etiological factor "confiscated" a frequent (5.7%) anthropogenic cause of capture for patients of WRBC. Illegal hunting continues to be a serious problem in the country, for both migratory and native species. Although, the scale of this type of crime cannot be compared to that in Lebanon, for example, where about two and a half million birds are killed illegally every year (Raine et al., 2021), 15% of the anthropogenic causes of incidents with White Stork (*Ciconia ciconia*) are due to illegal shooting.

Although it is difficult to determine the exact number of birds injured by vehicle collisions, it is estimated that 2 to 9 million birds are killed on the road in Europe annually, with numbers varying between countries (Vidal-Vallés, 2018). In Bulgaria, 11.3% of human-related reasons for admission of injured storks are the result of a collision with vehicles.

Other anthropogenic factors include causes with a very small number of cases, but significant from the point of view of the impact of human activity, such as poisoning, entanglement in a nylon string or fence, falling into machine oil, collision with a wind turbine, or a destroyed nest due to repair work.

A thorough analysis of the accidents with the admitted White Storks (*Ciconia ciconia*) by month shows that the highest percentage of incidents occur in the months of June, July and August (Figure 2). The elevated death rate in the period from April to May is related to increased mortality of the young during the nestling period and the temperature changes. Research of Jovani & Tella (2004) shows that 91% of deaths occurred on nestlings below 20 days of age, 73% concentrating on nestlings up to 10 days-old, when the thermoregulation of the birds is not well developed and this coincides with the period of spring rainfall. Another reason for these high values appear to be the inexperienced attempts of the young birds to fly from their nests, which makes them more susceptible to accidents (Harness & Wilson, 2001).

The highest percentage of injured individuals was observed in the month of July (34.1%), which is associated with the lack of experience of the young birds during their post-nesting period. During the beginning of the migration and hunting season, the admittance of shot birds increases due to the high percentage of incidents in the month of August, 31.5% of the entire annual intake. In a study conducted in 2004 in southern Bulgaria, a fatal outcome was found in most cases with storks taken in due to collision with power lines; this study found an increase in mortality after collision with power lines, mainly during migration and roosting in large flocks (Demerdzhiev et al., 2009). Storks gather in large flocks in preparation for flight, and their gathering and resting places are often close to power lines, and the crowding of many individuals in limited areas forces the birds to land on power lines and increases the risk of accidents (Demerdzhiev, 2014; Gradev et al., 2012). The explanation of the high values found by us in the victims of electricity in August at the beginning of the migration process is also due to the so-called bottleneck territories along the migration route. It is known that during flight, when birds reach natural barriers (e.g. mountain, sea, etc.), their migration is concentrated on a limited strip of land (e.g. Burgas Bay, Bosphorus, etc.) (Gerdzhikov et al., 2014.). In the same way, the migration pattern in northeastern Bulgaria can be explained, where in the relatively flat topography white storks fly on a wider front and in smaller flocks compared to the barrier in Burgas Bay, southeastern Bulgaria (Gerdzhikov et al., 2014). The presence of a lot of agricultural land and open grassland habitats is also relevant to the high casualty rates, with the lack of tall trees leading to the use of electric poles as perches by the birds, as well as the fact that raptors use these habitats as hunting grounds (Gerdzhikov & Demerdzhiev, 2009).

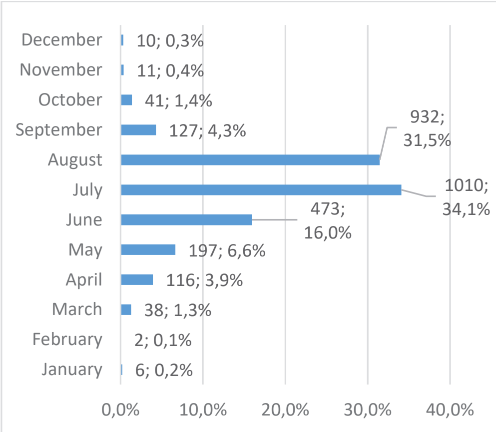

Figure 2. Monthly admissions to the Wildlife Rehabilitation and Breeding Centre “Green Balkans” for White Storks

The analysis of injured storks admitted to WRBC, distributed by age, shows that 64.4% of all accepted birds of the species were juveniles. The main reason for the incidents in this age group (41.0%) (Table 1) is falling from the nest during their first flight attempts. A study in Poland shows that early post-fledgling mortality rate varied from 2 to 11% (mean 4.3%) of all fledglings per year, with 73% dead birds found less than 100 m from their nests (Tobolka, 2014). On the other hand, the increasing use of plastic (polypropylene) string results in a large number of adult white storks carrying pieces of it into the nest. When moving, stork chicks can become entangled in the free ends of such strings, which, as the bird grows, often leads to limb necrosis (Kwieciński et al., 2006). It is the most common cause of injury in the category of "Other anthropogenic factors" in young birds admitted for treatment at WRBC.

In adult birds, where the main anthropogenic factor is again electricity, a prerequisite for accidents is the fact that the large wingspan creates the potential for contact between two wires and the creation of a voltage arc, as a result of which they become a victim of electricity (Stoychev & Karafeisov, 2003). The birds' use of electric poles as a resting place increases the risk of poaching due to them being easily spotted by hunters. In certain terrains, electric poles are the only possible resting place for the birds, but their location near roads makes them an easy target for shooting. However, such observed cases are few and the number of gunshot patients is 1.3%.

When discovering fallen nests or nests built on old buildings, in adverse weather conditions or for safety reasons, it is necessary to take the eggs. Such eggs are incubated at WRBC and represent 0.6% of all incoming patient cases of the species.

Table 1. Distribution of white storks admitted for treatment at the WRBC by etiology and age

AGE	egg n/% of total	juveniles n/% of total	immaturus n/% of total	adult n/% of total
Infection disease	0/0.0%	2/0.1%	0/0.0%	2/0.3%
Rivalry	0/0.0%	6/0.2%	2/0.1%	6/0.2%
Other anthropogenic factors	8/0.3%	50/1.7%	16/0.5%	12/0.4%
Electrocution and collision with power line	0/0.0%	29/1.0%	17/0.6%	36/1.2%
Confiscated	0/0.0%	6/0.2%	6/0.2%	2/0.1%
Fallen from a nest	0/0.0%	1214/41.0%	73/2.5%	13/0.4%
Unknown	0/0.0%	485/16.4%	318/10.7%	466/15.7%
Gunshot	0/0.0%	8/0.3%	3/0.1%	26/0.9%
Collision with vehicles	0/0.0%	9/0.3%	9/0.3%	10/0.3%
Extreme weather	10/0.3%	100/3.4%	2/0.1%	17/0.6%
Total	18/0.6%	1909/64.4%	446/15.1%	590/19.9%

In our study, the analysis of rehabilitation outcomes showed an overall discharge rate of 43.1% of hospitalised Storks (Table 2). This rate corresponds to data from the total number of rehabilitated patients of other centres in Great Britain (40%) (Grogan & Kelly, 2013) and lower than the total success of fully recovered patients in centres in Spain - 50% (Molina-Lopez et al., 2017). The "Fallen from nest" category represents the most favourable prognosis for survival and recovery back in the wild (24.9%). The survival of juveniles of all species is the same and that this age group is released more than 50% in a rehabilitation centre (Ress & Craig, 2004). Juveniles usually have less injuries and the goal is to feed and grow them without habituation with humans until they are ready for release and independent survival in the wild.

Table 2. Final outcome for White storks admitted to WRBC

Outcome	Captivity	Euthanasia	Lethal	Released
Infection disease	0/0.0%	1/0.0%	2/0.1%	1/0.0%
Rivalry	0/0.0%	1/0.0%	10/0.3%	3/0.1%
Other anthropogenic factors	3/0.1%	18/0.6%	37/1.2%	28/0.9%
Electrocution and collision with power line	3/0.1%	27/0.9%	41/1.4%	11/0.4%
Confiscated	0/0.0%	1/0.0%	1/0.0%	12/0.4%
Fallen from a nest	26/0.9%	156/5.3%	379/12.8%	739/24.9%
Unknown	75/2.5%	294/9.9%	486/16.4%	414/14.0%
Gunshot	2/0.1%	2/0.1%	27/0.9%	6/0.2%
Collision with vehicles	0/0.0%	6/0.2%	18/0.6%	4/0.1%
Extreme weather	2/0.1%	8/0.3%	60/2.0%	59/2.0%
Total	111/3.7 %	514/17.3 %	1061/35.8 %	1277/43.1 %

For 35.8% of the white storks admitted to the WRBC, despite rehabilitation efforts, the injuries were severe and ended fatally. High mortality is observed mostly in storks injured by electrocution and collision with power lines. Because death in these types of injuries is often

instantaneous, a large proportion of these patients were found dead. Those who made it out alive were usually in poor condition with severe disabilities. Birds may survive the initial injury and recover or die later from complications. Due to the lack of any chance of survival, euthanasia was required in 17.3% of the injured individuals. In 3.7% of the incidents with white storks, independent survival in nature was impossible, as a result of which they became part of reintroduction programs in zoos, visitation and reintroduction centres.

CONCLUSIONS

Despite the legal regulations in place, anthropogenic activities continue to be a threat to the white stork with a number of offences and wildlife crimes. Wildlife rehabilitation centres are structures that can support both the survival of populations and the monitoring of changes in them.

Corroborating a number of other studies, the analysis of WRBC admissions data shows that young birds are most vulnerable during the breeding season due to temperature changes during this season, as well as unsuccessful first flight attempts of the young ones.

The power grid poses potential risks to populations of protected bird species. The specific location of Bulgaria in relation to international migration routes makes it an important factor for the survival of migratory species.

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THE INFLUENCE OF REARING CONDITIONS ON GROWTH, MEAT QUALITY AND MORTALITY OF *Acipenser ruthenus*

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Abstract

To evaluate the growth of sterlet sturgeon (Acipenser ruthenus, Linnaeus 1758) in different rearing production systems (recirculating aquaculture system - RAS and earthen ponds system), the meat quality (biochemical profile), growth (allometric and Fulton coefficient) and mortality indices were determined. Sterlet sturgeon specimen were reared for 60 days in a RAS system, after which half of the biological material was transferred into earthen ponds, while the other half was further reared in the RAS system. The physico-chemical parameters of the technological water were monitored during the experimental period. The fish specimens were fed by using the same feed and the same feeding strategy. It was observed that the specimen reared in earthen ponds presented an isometric growth, while those reared in the RAS system had a positive allometric growth. The results registered after biochemical fish meat analysis highlighted that specimen reared in earthen ponds had a higher protein concentration compared to the ones reared in RAS system. As well, the survival rate of fish individuals was higher in the earthen ponds system.

Key words: *allometry, aquaculture systems, Fulton, meat biochemistry.*

INTRODUCTION

Due to the lack of bones, with a palatable meat and being a source of black caviar, sturgeons are considered a high value product.

Sterlet (*Acipenser ruthenus*) belongs to the *Acipenseridae* family, is a species relatively small compared with other members of the family.

It is a species with rapid sexual maturation. In a controlled environment, sturgeons grow faster and reach sexual maturity earlier, compared to a natural environment (Chebanov & Billard, 2001).

Sterlet is an endangered species in the wild, due to dam construction across the rivers, over-fishing, pollution, and the degradation of the natural environment (Billard & Lecointre, 2001). All these factors are claimed to disturb the migratory routs, affecting the reproduction potential of the sterlet (Heidary et al., 2012). One alternative solution to this dramatic decline in stocks could be represented by aquaculture.

Advances in nutrition and the management of the production cycle could improve the economic efficiency of the aquaculture industry. (Hassaan et al., 2019)

This study aimed to present the differences (length-weight correlation, biochemical profile of the meat, survival rate) between sterlet reared under controlled conditions in a recirculating aquaculture system and sterlet reared in floating cages under natural environmental conditions, while also comparing our data with relevant data from specialised literature.

Based on the presented data, adjustments can be made in order to optimise the technological parameters for rearing sterlet, with the goal of obtaining high quality fish products, valuable for human consumption.

MATERIALS AND METHODS

Sterlet sturgeon specimen were reared for 30 days in a RAS system, from fecundated roes, up to the size of 192±29 mm and 2.09±0.6 g. After those 30 days, half of the fish fry were transferred into earthen ponds, while the other half were further reared in the RAS system for another 60 days, which represents the total experimental period.

In the RAS system, 900 fish fry were equally stocked in 3 experimental fiberglass tanks (1.4×1.4×0.4 m), coded T1 (tank 1), T2 (tank 2) and T3 (tank 3). Also, in the earthen ponds, 900 fish fry were equally stocked in 3 floating cages (4×4×2 m), coded FC1 (floating cage 1), FC2 (floating cage 2) and FC3 (floating cage 3). The frame is constructed from double HDPE Φ = 200 mm. A growth enclosure made of polyamide (PA) mesh was used to line the inside of the floating cage. The size of the 7 mm eye of the mesh was chosen both to prevent the escape of sterlet, as well as to prevent other species from entering the cage, which could have competed for food.

During the experimental period, in the RAS system, compressed air was used through an air stone in each tank, in order to continuously provide water aeration.

The sterlet was reared under natural photoperiod in the RAS system as similar as possible to the earthen ponds.

Throughout the experiment, water temperature and dissolved oxygen concentrations, were recorded daily, in both the experimental tanks and floating cages.

Weakly, the water parameters presented in Table 1 were monitored in the RAS system and the floating cages, using kits and the corresponding methods for analysis.

Ammonia was determined using Seignette salt and Nessler reagent, based on pH level. The values are presented as average ± standard deviation.

Table 1. Average values for monitored water parameters during the experimental period

Parameter	Unit	Tank 1	Tank 2	Tank 3	Floating Cage 1	Floating Cage 2	Floating Cage 3
pH	upH	7.91±0.15	7.93±0.14	7.96±0.19	7.65±0.32	7.26±0.11	7.39±0.18
Organic matter	mg $KMnO_4$/l	25.12±13.95	25.99±12.03	24.63±6.57	46.01±2.83	52.18±7.99	38.31±1.02
CCO-Mn	mg O_2 /l	6.35±3.53	6.28±2.90	7.49±1.49	19.23±1.18	18.14±1.10	17.16±1.85
Calcium Ca^{2+}	mg/l	53.10±5.14	56.11±6.06	49.48±8.82	60.12±6.11	56.11±4.86	64.08±7.27
Magnesium Mg^{2+}	mg/l	27.95±5.36	23.13±5.59	26.38±6.74	41.31±2.55	31.87±2.19	39.16±4.37
Ca^{2+}/Mg^{2+}	-	1.90	2.43	1.88	1.45	1.76	1.63
Total hardness	ºD	12.63±2.95	12.72±1.71	12.73±0.81	17.9±1.88	14.02±1.97	12.9±1.63
Nitrites NO^{2-}	mgN/l	0.03±0.06	0.02±0.07	0.01±0.06	0.01±0.01	0.01±0.01	0.01±0.01
Nitrates NO^{3-}	mgN/l	2.01±1.52	2.32±1.40	2.46±1.74	0.86±0.18	0.31±0.15	0.35±0.18
Chlorides Cl^-	mg/l	25.93±5.24	25.48±4.20	24.57±6.35	12.76±3.22	12.76±2.59	12.76±2.80
Ammonium NH^{4+}	mgN/l	0.36±0.30	0.26±0.27	0.39±0.17	0.25±0.13	0.25±0.09	0.25±0.10
Ammonia NH_3	mgN/l	0.01±0.01	0.01±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01

Average ± standard deviation

The phytoplankton and zooplankton profiles of the water were analysed in the earthen pond where the floating cages were placed, in order to assess the present species (Table 2).

Table 2. Phytoplankton and zooplankton present in the water of the earthen pond

	Unit	Value	Systemic group, %		
Phytoplankton			Chlorophyta	Cyanophyta	Bacillariophyta
Density	no./m^3	1471.69	8	5.5	86.5
Biomass	g/m^3	378.58	1.5	1.75	96.75
Zooplankton			Rotatoria	Copepoda	Cladocera
Density	no./m^3	19	11	71.25	17.5
Biomass	g/m^3	5.55	1.5	77.75	20.75

Fish feed was provided 3 times per day to the fish, using a commercial diet for this species, with a feeding rate of 2% from total fish biomass. The same fodder and feeding rate were used for all the tanks and floating cages.

The weight (g) and length (cm) of 50 individual fish specimen, at the start and the end of the experiment, were recorded. The data is presented for each tank and floating cage as average ± standard deviation.

Ten fish specimens were sampled from each experimental tank and floating cage in order to perform the proximate analysis for the meat. Water content, total lipids, crude protein, and ash were determined using standard methods AOAC (1995).

Fish were anesthetized before sampling by bathing for 5 minutes in a clove oil solution, in order to abide to law no. 43/2014 on the protection of animals used for scientific purposes and Directive 2010/63/EU of the European Parliament and of the Council from 22nd September 2010 on the protection of animals used for scientific purposes.

The correlation between fish length and weight is described using the equation:

$w = a \times l^b$,

where:

w - fish weight, g;

l - fish length, cm;

a - constant equal to w when l = 1;

b - exponential/allometric coefficient.

When the rearing process of the fish has a harmonic balance between length and weight, the exponential coefficient "b" is considered equal to 3 (isometric growth). When b>3, the fish growth is described as having a positive allometry, and at the opposite pole, when b<3 the growth of the fish has a negative allometry (the fish grows more in length than in weight) (Bulat, 2017)

Fulton coefficient (K, %), that represents the fish condition factor, was determined using the formula:

$K (\%) = \text{Weight (g)}/\text{Standard body length (cm)}^3 \times 100$

The survival rate was measured using the formula:

Survival rate (%) = $(N_t/N_0) \times 100$,

where:

N_t - final number of fish,

N_0 - initial number of fish.

The data was submitted to one-way classification variance analysis and ANOVA test was used, to highlight statistical differences. When differences were statistically significant ($p \leq 0.05$), Tukey's post hoc test was applied to identify which group differs. The data registered in this study was compared with data from aquaculture and wild ecosystems that are published in referenced journals.

RESULTS AND DISCUSSIONS

Water parameters had values, both in the experimental tanks and in the floating cages, that fell within the accepted range for sterlet at this stage of developmental growth (Table 1).

The pH registered slightly lower values in the floating cages, with a higher organic matter concentration in the floating cages. Nitrogen compounds had values within the limits approved by Order no. 161/2006 emitted by the Ministry of Environment and Water Management, regarding the ecological status and quality of surface waters (Order no. 161/2006).

The length-weight correlation, at the end of the experiment, in the tanks from RAS, is represented in Figure 1. It can be observed that the regression coefficient b is higher than 3 in all experimental tanks, which suggests that fish had a growth focused on weight gain. The highest allometric growth was achieved in tank 2, the differences between the experimental tanks T1 and T3 were not statistically significant ($p > 0.05$).

In an experiment done by Prokeš et al. (2011), sterlet reared under experimental conditions reached a regression coefficient b = 3.4834, for fish of similar age and size.

Lee et al. (2014), after administering an experimental fish feed supplemented with garlic powder for 12 weeks, obtained sterlet fingerlings with average weight ranging between 25.64±1.12 and 30.18±0.21 g. These values are higher than those presented in the present paper, but considering the length of our experiment, it can be assessed that our sterlet specimens would reach similar sizes after an equal amount of time.

The Fulton's condition factor had values raging between 0.47±0.01 (T2) and 0.49±0.03 (T1). These values are comparable to those obtained by Prokeš et al. (1996) for sterlet at 170.9 mm and 22.8 g (0.45-0.37).

In the floating cages, the length-weight correlation was characterised by a negative growth, the fish growing rather in length than in weight, as in the tanks from the recirculating aquaculture system. The regression coefficient b had values between 2.9887 and 2.9954, without significant differences between FC1 and FC2 ($p > 0.05$), but FC3 had significant differences between the other two experimental variants ($p < 0.05$).

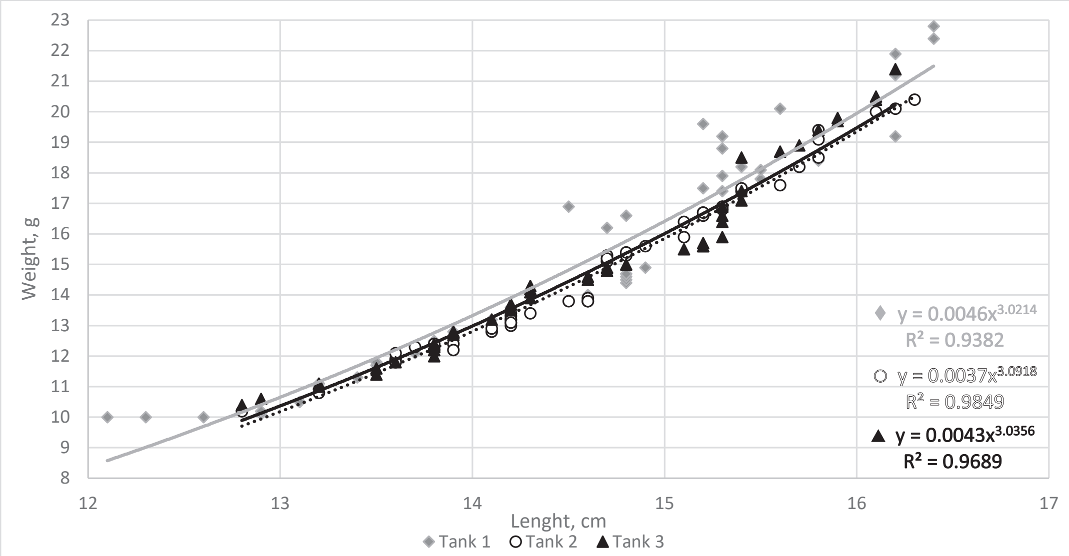

Figure 1. Length-weight correlation in sterlet reared in three tanks of a recirculating aquaculture system, for 60 days

In Figure 2, the length and weight are presented, as well as the correlation between these two parameters.

Rybnikár et al. (2011) observed a negative growth in sterlet reared from day 64 to day 72 after hatching, for fish of similar size.

The condition factor for sterlet reared in floating cages in our experiment, had values between 0.53±0.03 (FC2) and 0.55±0.01 (FC3), values comparable to what Rybnikár et al. (2011) measured in the paper mentioned above.

In an experiment done by Wiszniewski et al. (2019), sterlet fed with feed supplemented with bromelain, had a final condition factor (0.42±0.03) lower than the condition factor for the sterlet reared during experiment presented in this paper.

The average fish length in each tank is as follows: 14.51±1.16 cm (T1), 14.64±0.80 cm (T2) and 14.58±0.89 cm (T3). In the floating cages, the average length for sterlet was 13.31±0.54 cm in FC1, 13.39±0.60 cm in FC2 and 13.32±0.63 cm in FC3. These values did not have statistically significant differences between tanks or floating cages ($p > 0.05$), but between experimental variants, the differences were significant ($p < 0.05$).

Regarding the average weight of the sterlet fingerlings, in the tanks these values were: 15.14±3.65 g for T1, 14.86±2.58 g for T2 and 14.86±2.85 g for T3. There were significant differences between tank 1 and the other two tanks ($p < 0.05$). Sterlet reared in the floating cages, measured an average weight of 12.66±1.63 g in FC1, 12.80±1.96 g in FC2 and 13.19±1.92 g in FC3. The differences between FC3 and the other two floating cages were statistically significant ($p < 0.05$).

These differences in final average weight between tanks or floating cages can be explained by a lower fish density. The higher weight in both T1 and FC3, where there were significant differences, corresponds to lower final fish densities (Table 3).

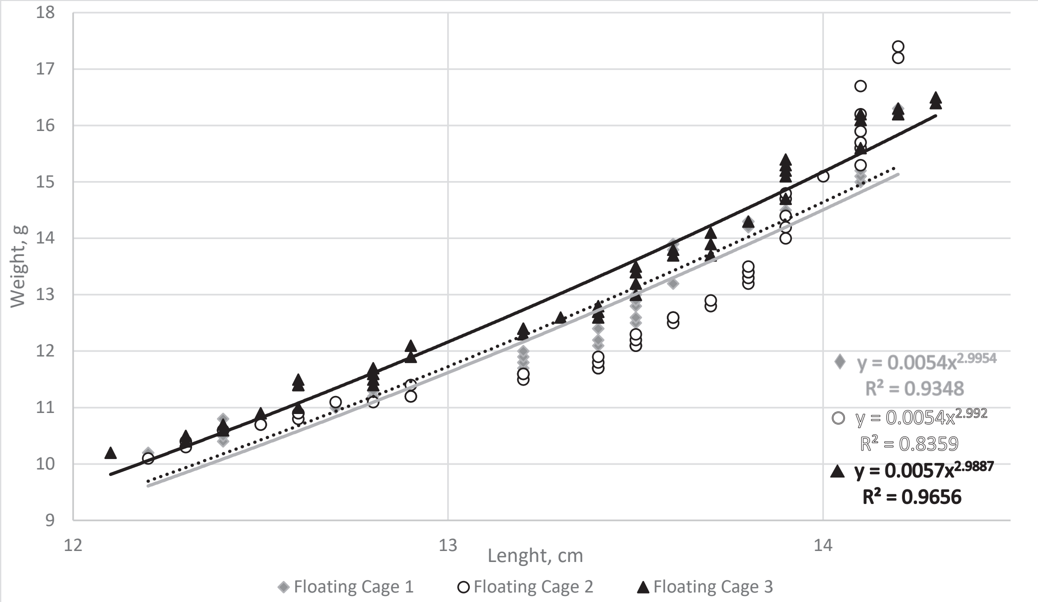

Figure 2. Length-weight correlation in sterlet reared in three floating cages from an earthen pond, for 60 days

In this experiment, the macronutrients from the meat of the fish were also determined.

At the start of the experiment, the average percentage of water in meat was 92.89±0.73%.

After 60 days, the average water quantity reached values between 78.02±1.18% (T1) and 78.41±0.53% (T2) in the tanks from the recirculating aquaculture system, with significant differences between T1 and T2 ($p < 0.05$).

In the floating cages, after the same period, water represented between 77.51±0.49% (FC2) and 77.72±0.12% (FC3) in the meat of sterlet, values without significant differences ($p > 0.05$).

Between the two experimental variants, the differences were statistically significant ($p < 0.05$). Figures 3 and 4 present the graphical representation of these changes in water quantity from the meat of sterlet.

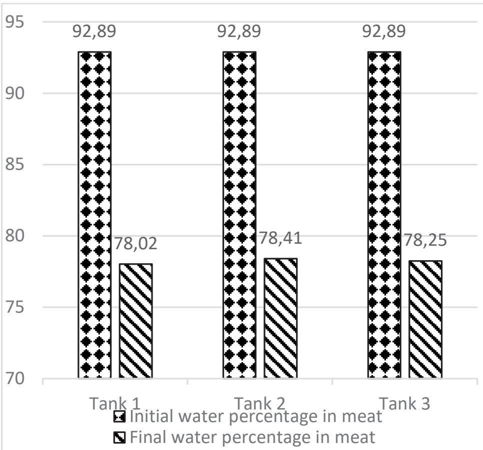

Figure 3. Water percentage in the meat of sterlet reared in tanks from a recirculating aquaculture system, at the start and end of the experiment

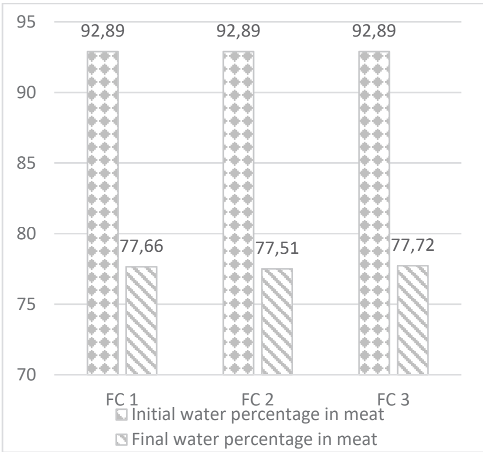

Figure 4. Water percentage in the meat of sterlet reared in floating cages from an earthen pond, at the start and end of the experiment

The initial protein quantity in meat, at the start of the experiment, represented 5.82±0.92%.
At the end of the experiment, the highest protein percentage in the tanks, was measured in T1 (13.48±0.22%) and the lowest protein percentage in T2 (13.05±0.76%), with significant statistical differences between T1 and the other two tanks ($p < 0.05$).

In the floating cages, the highest value was obtained in FC2 (14.05±0.5%) and the lowest protein percentage was measured in FC3 (13.72±0.83%). Between the experimental variants, the differences were statistically significant ($p < 0.05$).
Figure 5 and 6 present the data measured for protein percentage in the meat of sterlet.

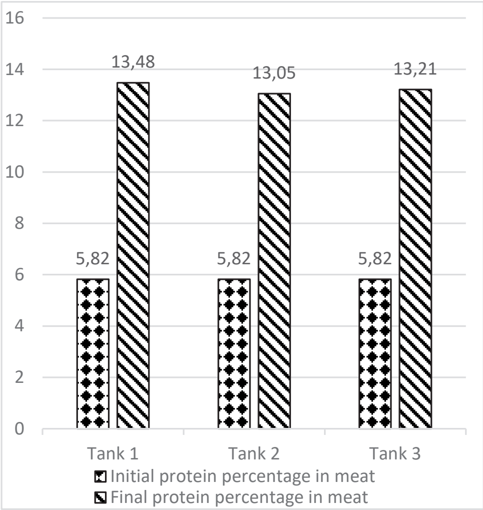

Figure 5. Protein percentage in the meat of sterlet reared in tanks from a recirculating aquaculture system, at the start and end of the experiment

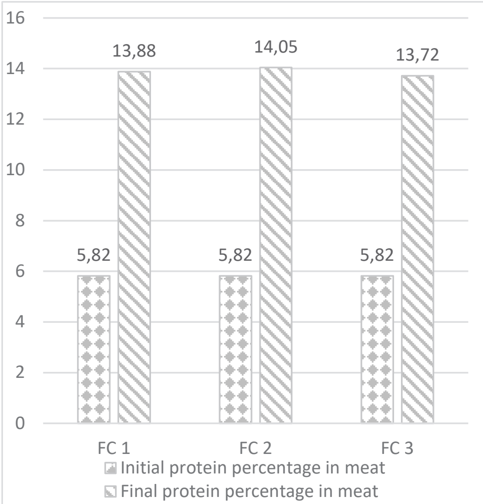

Figure 6. Protein percentage in the meat of sterlet reared in tanks from a recirculating aquaculture system, at the start and end of the experiment

Lipid percentage had the highest increase among the macronutrients. Starting with 0.44±0.08% lipids in the meat, sterlet accumulated, during the experimental period, up to 17 times more lipids. In the experimental tanks, lipids measured in meat varied between 7.49±0.22% (T2) and 7.55±0.38% (T1), without significant differences ($p > 0.05$). In the floating cages, the values for lipids measured in meat, were between 7.33±0.15% (FC2) and 7.47±0.09% (FC3), with significant differences between FC2 and FC3 ($p < 0.05$).
The variation of lipids percentage in sterlet meat, is presented in Figures 7 and 8.
Lipid content in fish meat from the tanks is different from that of fish reared in floating cages. This can be explained by the various behaviours that require various expenditures of energy, these factors cause an increased fat content in the tissue of sterlet (Ovissipour & Rasco, 2011; Ghomi et al., 2013).
The percentage of ash, presented in Figures 9 and 10, found in the meat of sterlet at the start of the experiment, had a value of 0.68±0.02%. At the end of the experiment, the ash represented 0.83±0.01% (T1 and T3) in the sterlet meat. The lowest average value was measured in T2 (0.81±0.01). Values in the tanks had no significant statistical differences ($p > 0.05$). The floating cages had an average value of ash, slightly higher than those obtained in tanks, with 0.85±0.01% in both FC1 and FC2, and the lowest value in this experimental variant was 0.82±0.01% in FC3.

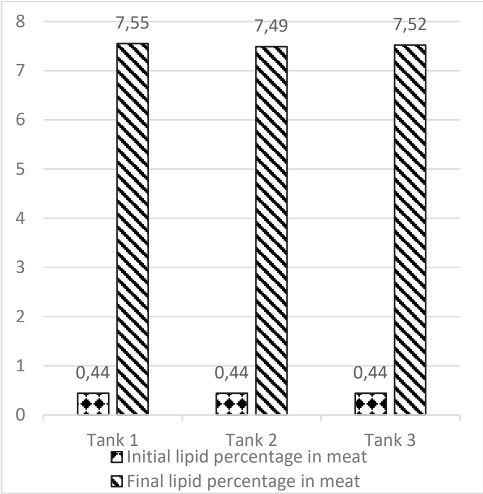

Figure 7. Lipid percentage in the meat of sterlet reared in tanks from a recirculating aquaculture system, at the start and end of the experiment

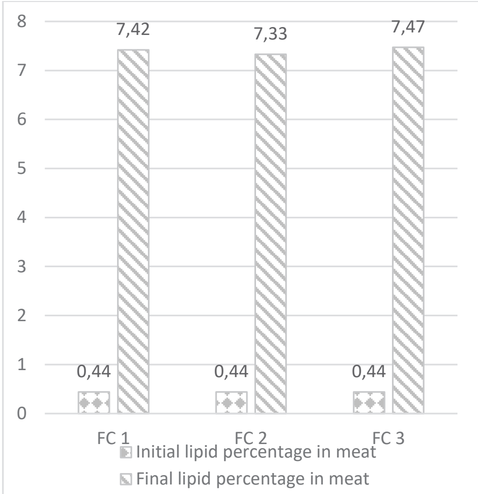

Figure 8. Lipid percentage in the meat of sterlet reared in tanks from a recirculating aquaculture system, at the start and end of the experiment

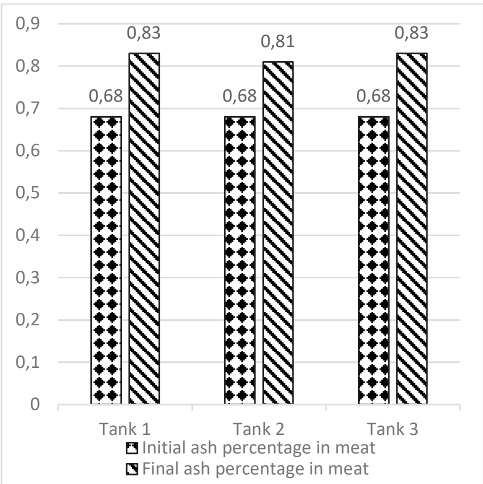

Figure 9. Ash percentage in the meat of sterlet reared in tanks from a recirculating aquaculture system, at the start and end of the experiment

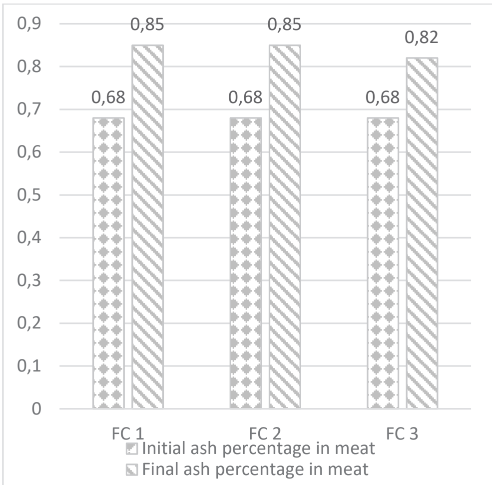

Figure 10. Ash percentage in the meat of sterlet reared in tanks from a recirculating aquaculture system, at the start and end of the experiment

In Figure 11, the survival rate of sterlet reared in tanks is represented as number of fish deaths per week, for each tank. Each tank was populated with 300 fingerlings, and at the end of the experiment there were 193 specimens in T1, 198 specimens in T2 and 199 specimens in T3. The survival rate for each tank was as follows: 64.33% in T1, 66% in T2 and 66.33% in T3.

For sterlet reared in floating cages, the survival rate was higher and reached 75.66% in FC1, 74.66% in FC2, and 72% in FC3. The final number of fingerlings that survived in the floating cages was: 227 specimens in FC1, 224 specimens in FC2, and 216 specimens in FC3. In Figure 12, the survival rate of sterlet reared in floating cages is represented as number of fish deaths per week, for each floating cage.

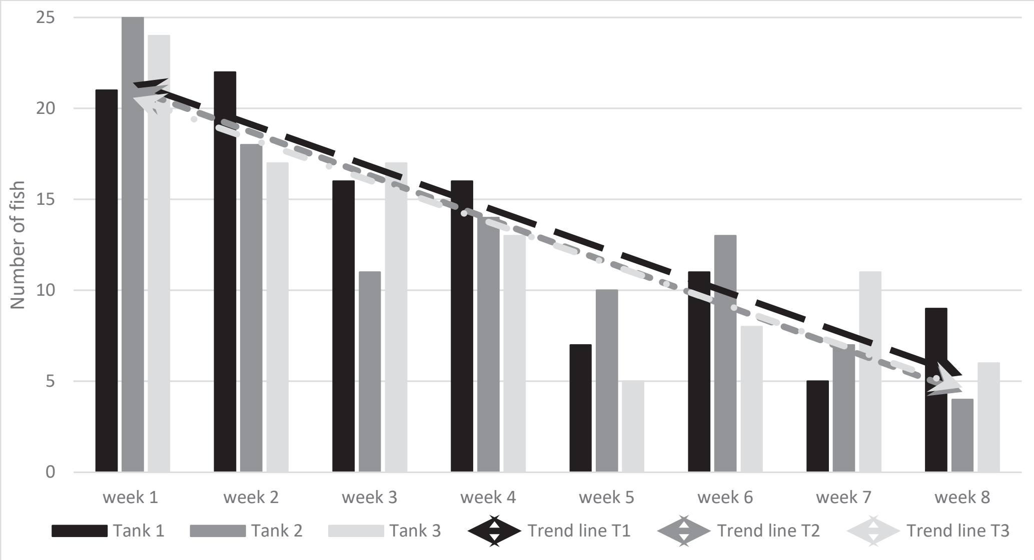

Figure 11. Number of fish deaths per week in tanks from recirculating aquaculture system, during the experimental period

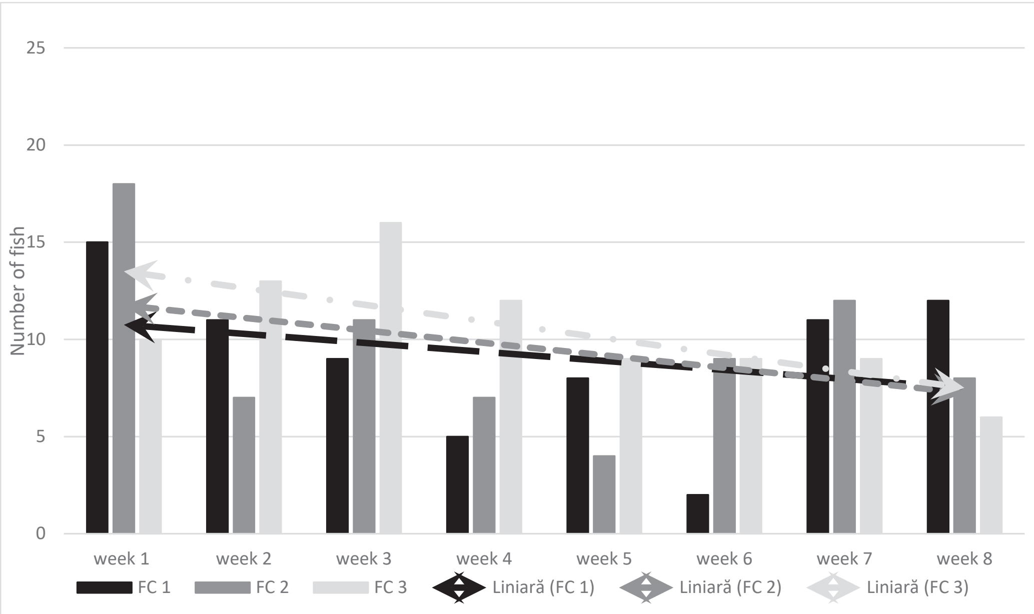

Figure 12. Number of fish deaths per week in floating cages from an earthen pond, during the experimental period

The differences in macronutrient accumulation and survival rate can be attributed to the influence of the rearing environment. The floating cages, being placed in an earthen pond, were subjected to the elements, while the tanks were part of a recirculating aquaculture system, a closed system rigorously monitored.

In the floating cages, sterlet fingerlings had, besides the administered fish feed, a natural source of nutrients represented by zooplankton organism such as Copepoda, Cladocera and Rotatoria. One of the most important elements in regard to the technology applied in intensive aquaculture, is represented by the administered

fish feed. Fish tissue is significantly shaped by feed quality, recipe and method of delivery. (Chipinov et al., 2012)
Therefore, sterlet reared under intense aquaculture conditions with just artificial feed leads to changes in the physiology and biochemistry in the bodies of the fish, compared to fish from natural environment. (Kireyev, 2011)

Stocking density also has an impact on the growth and survival of individuals, the floating cages having a much larger surface area and water volume. In Table 3, the stocking and final densities are presented as number of fish per unit of surface and unit of volume, for each experimental variant.

Table. 3 Fish densities in the rearing units during the experimental period

	Unit	Tank 1	Tank 2	Tank 3	Floating cage 1	Floating cage 2	Floating cage 3
Stocking density per surface	No. fish/ m^2	153	153	153	18.8	18.8	18.8
	kg/m^2	0.320	0.320	0.320	0.039	0.039	0.039
Stocking density per volume	No. fish/ m^3	382.7	382.7	382.7	9.4	9.4	9.4
	kg/m^3	0.800	0.800	0.800	0.020	0.020	0.020
Final density per surface	No. fish/ m^2	98.5	101	101.5	14.2	14	13.5
	kg/m^2	1.491	1.501	1.509	0.180	0.179	0.178
Final density per volume	No. fish/m^3	246.2	252.6	253.8	7.1	7	6.75
	kg/m^3	3.727	3.753	3.772	0.090	0.090	0.090

CONCLUSIONS

Artificial rearing technologies of fish are dependent of the rearing systems and must consider the most optimal and economically efficient path to high production and safe-for-consumer fish products.
From our productional rearing results, sterlet reared in a recirculating system has a higher growth potential, with a better use of nutrients from the feed, but sterlet reared in earthen ponds has a better protein percentage with a higher survival rate.
Optimizing the fish feed and rearing conditions to increase the biological value of sterlet, will result in a better survival rate. Taking these measures, rearing sterlet could have a higher economic outcome with an improved nutritional value of the meat.

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EVALUATION OF THE HAEMATOLOGICAL PROFILE AND BIOCHEMICAL INDICES IN THE BLOOD OF COMMON CARP (*Cyprinus carpio*), AS RESPONSE TO SUPPLEMENTING THEIR DIET WITH PHYTOGENIC COMPOUNDS

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Abstract

The aim of this study was to evaluate the impact of different phytogenic compounds on the haematological profile and biochemical indices of carp, reared in a recirculating pilot aquaculture system. The experiment was conducted for 52 days. A basic feed, Aqua Classic type, with 46% protein and 22% lipid, was used. 5 g of different phytogenic compounds were added to form the experimental diets, as follows: V1 - control, V2 - 0.5% licorice (Glycyrrhiza glabra), V3 - 0.5% echinacea (Echinacea purpurea) and V4 - 0.5% wild thyme (Thymus serpyllum). At the end of the experiment, blood samples were taken to evaluate the haematological and blood biochemical parameters. The mean values for Ht, Hb, RBC and WBC were higher in the experimental variants than in the control. Serum protein was significantly lower ($p<0.05$) in variants V2 and V3 compared to the control. The serum glucose values registered significant differences between the control and the 3 experimental variants. In conclusion, the addition of phytogenic compounds in the diet has beneficial effects on the haematological and blood biochemical profile of carp (Cyprinus carpio).

Key words: *aquaculture, carp, phytogenic, haematological profile.*

INTRODUCTION

Due to the high-quality proteins and lipids, it contains, fish are recognized as some of the healthiest foods, contributing to a balanced human diet. According to the FAO, fish provided about 17% of the animal protein consumed worldwide in 2017 (FAO, 2020).

Intensive or super-intensive fish farming is practiced to respond effectively to consumer demand. High fish densities per spawning unit lead to a higher incidence of stress-induced diseases and reduced immunity.

In intensive aquaculture, nutrition is one of the most important aspects. Feed quality in aquaculture is an important condition to be met (Iheanacho et al., 2017). The quantity and quality of the feed provided determines the proper growth and development of the reared fish species. Optimizing the amount of feed improves carcass biochemistry, good feed conversion and protection of fish from pathogens (Volkoff et al., 2010; Singha et al., 2020). Food can affect water quality, can help to increase the fish's resistance to disease, and the nutrients it contains can increase productivity (Heal et al., 2021; Hassaan et al., 2020).

In recent years, due to the decline of wild fish stocks, it is desirable to replace fishmeal in feed with herbal substitutes, to introduce by-products of the food industry and even medicinal plants rich in bioactive compounds (Adekoya et al., 2018; Savin et al., 2022).

There is a need to develop diets with natural and inexpensive ingredients capable of improving the physiological and biological functions of fish for high growth and better disease resistance (Salam et al., 2021).

Plant extracts rich in phytogenic compounds have been shown to improve the growth performance and immunity of aquatic animals and increase their resilience to adverse environmental conditions (Ahmadifar et al., 2020; Adel et al., 2021; Magouz et al., 2021).

The present study was conducted to determine the effect of adding phytogenic compounds to the diet of carp (*Cyprinus carpio*) on the haematological profile and its biochemical indices.

MATERIALS AND METHODS

The experiment was conducted at the Institute of Research and Development for Aquatic Ecology, Fishing and Aquaculture Galati for 52 days in a recirculating system consisting of 4 glass aquariums, with dimensions of 100 x 40 x 25 cm and a water volume of 100 liters. Each breeding unit had a water treatment system, represented by Tetra EX 1200 Plus external filters with 5 different filter media: ceramic rings, bio balls, sponge, carbon filter and felt.
185 specimens of carp (*Cyprinus carpio*) with an average mass of 40.5±0.31 g obtained from the Experimental Laboratory of Agro-Fisheries Research Brates were randomly distributed in the rearing units. They were acclimated for 7 days during which they were fed the commercial Aqua Clasic diet with 2 mm granulation, a protein content of 46% and a fat content of 22%, purchased from Aqua Garant (Table 1).

Table 1. Structure and nutritional value of basic carp feed

Parameters	***Quantity***
Crude protein	46 %
Crude fat	22 %
Cellulose	1.5 %
Calcium	1.5 %
Sodium	0.35 %
Phosphorus	1.15 %
Vitamin A	10000 U.I.
Vitamin C	250 mg
Vitamin E	200 mg

The feed ration used was 2.5%/day of body weight and food was administered twice a day. Three experimental diets and one control diet were prepared:
- Control diet (AM) - without phytogenic compounds;
- Diet 1 (A1) - with the addition of 5 g of licorice (*Glycyrrhiza glabra*) to 1 kg of basic feed;
- Diet 2 (A2) - with an addition of 5 g echinacea (*Echinacea purpurea)* to 1 kg of basic feed;
- Diet 3 (A3) - with the addition of 5 g of thyme (*Thymus serpyllum*) to 1 kg of basic feed.

The experimental diets were prepared according to the method described by Savin et al. (2022).

Blood samples were collected by tail vein puncture from 7 specimens of each experimental group using heparinized syringes to determine the haematological profile. Before blood sampling, the fish were anesthetized by bathing in a clove oil solution (2.5 ml per 10 litters of water).
According to Baker and Silverstone, 1976, red and white blood cells were counted in a Neubauer counting chamber after the blood had been diluted with Vulpian's solution for red blood cells and Turk's solution for white blood cells, respectively.
The formula used to determine the red blood cell (RBC) count was:

$$\text{RBC x } 10^6\ \mu l^{-1} = \text{n x } 10000,\ \text{n = number of erythrocytes in 80 squares}$$

White blood cells (WBC) were determined according to the formula:

$$\text{WBC x } 10^3\ \mu l^{-1} = \text{n x } 40,\ \text{n = number of white blood cells counted}$$

Haemoglobin (Hb, g dl^{-1}) was determined using the cyanmethemoglobin method described by Kondi (1981). 20 microliters of blood collected on anticoagulant was mixed with 5 ml of Drabkin's reagent and after 30 minutes the absorbance at 540 nm was read with a Spekol 1300 spectrophotometer (Analytik Yena).
Haematocrit (Ht, %) was determined by introducing blood into the capillary tubes and centrifuging horizontally in a microhematocrit centrifuge at 10,000 rpm for 2 minutes. It was expressed as a percentage, as a ratio of the erythrocyte's column to the whole blood column (Davison et al., 2023).
Based on the above, the erythrocyte constants (MCH, MCV, MCHC) were determined, according to the method described by Dacie & Lewis, 2011.
For protein and blood glucose determination, blood collected without anticoagulant was centrifuged at 4000 rpm for 5 minutes.
Total plasma proteins (TP, g dl^{-1}) were determined by the Biuret method according to Gornâll et al. (1949).
Blood glucose levels (GLU, mg dl^{-1}) were determined using the ortho-toluidine method described by Wedemeyer & Yasutake (1977).
Statistical analysis. For statistical analysis SPSS Statistics 17.0 for Windows was used. All data

are shown as mean ± standard deviation. The Kolmogorov-Smirnov normality test followed by the t-test was performed to check the differences between the experimental variants. Comparisons between variants were assessed using Duncan's test for multiple comparisons. The results were considered statistically different at $p<0.05$.

RESULTS AND DISCUSSIONS

Supplementation of the diet with phytogenic compounds significantly changed the haematological parameters of the fish in the groups that received diets supplemented with phytogenic compounds. Significant increases in haematocrit, haemoglobin concentration, and erythrocyte count were observed (Table 2).

The haematocrit (Ht) recorded significantly higher differences ($p<0.05$) in all the groups in which the phytogenic compounds were administered, the highest percentage being in the A3 variant (36.49±0.02), in which administered wild thyme. There were no significant differences ($p>0.05$) between the control group AM (34.81±0.50) and groups A1 (34.85±0.20) - licorice and A2 (35.19±0.01) - echinacea (Table 2).

Almarri et al., 2023 obtained similar results by supplementing the *Nile tilapia* diet with 0.5; 1; 1.5 and 2% *Annona squamosa* leaf extract. The increase in haematocrit in the groups fed with phytoadditives may indicate an improvement in the nutritional status of the fish.

Table 2. The effect of phytogenic compounds from licorice (A1), echinacea (A2) and wild thyme (A3) on haematological indices of *Cyprinus carpio*

Experimental diet	Haematocrit (%), mean ± sd	Haemoglobin (g/dl) mean ± sd	RBC ($*10^6/\mu l$) mean ± sd	WBC (* $10^3/\mu l$) mean ± sd	MCV (μm^3) mean ± sd	MCH (pg) mean ± sd	MCHC (g/dl) mean ± sd
AM	34.81±0.50	9.72±0.17	1.71±0.02	51.69±0.18	203.80±4.83	56.92±1.26	27.94±0.52
A1	34.85±0.20	9.81±0.02	1.92±0.02	52.07±0.13	181.65±1.88	51.16±0.44	28.16±0.16
A2	35.19±0.01	9.79±0.02	1.89±0.02	51.75±0.01	186.63±1.64	51.91±0.41	27.82±0.04
A3	36.49±0.02	9.83±0.08	1.90±0.02	50.73±0.04	191.91±1.93	51.70±0.76	26.94±0.20

Haemoglobin concentration increased slightly but not significantly ($p>0.05$) in groups A1, A2, and A3, respectively, compared to the AM control. The highest haemoglobin concentration was recorded in the group where wild thyme was administered, A3 (9.83±0.08), followed by the group with echinacea A2 (9.79±0.02), respectively the group where licorice was administered, A1 (9.81±0.02). The control group had the lowest haemoglobin concentration (9.72±0.17) (Table 2).

Compared to the control group, fish fed with phytogenic compounds had a significantly higher red blood cell (RBC) count ($p<0.05$) (Figure 1). The highest number of erythrocytes was in the case of fish from the group fed with licorice A1 (1.92±0.02), followed by A3 - wild thyme (1.90±0.02), A2 - echinacea (1.89±0.02), the lowest value being recorded in fish from the control group (1.71±0.02). Statistical analysis showed that there were also significant differences between A2 and A3 ($p<0.05$) versus A1, but no significant differences were found between A2 and A3 ($p=0.139$).

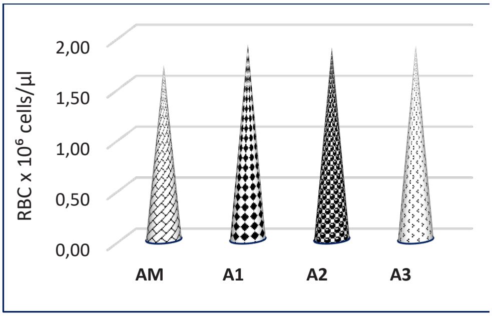

Figure 1. Variation in red blood cell count (RBC)

Regarding the number of white blood cells (WBC), there were insignificant differences ($p = 0.425$) between the control group and group A2, slightly increased in this group, in which the diet was supplemented with echinacea. In group A1 (52.07±0.13) the highest value of WBC was recorded, the differences being significant, compared to control group AM (51.69±0.18). Instead, the number of WBC decreased significantly in group A3 (50.73±0.04) compared to the control (Figure 2).

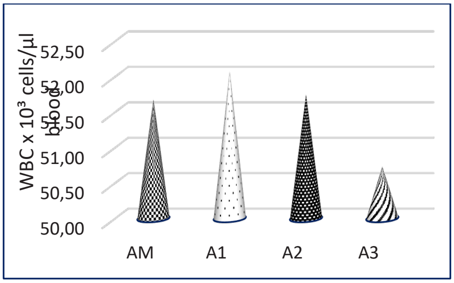

Figure 2. Variation in white blood cell count (WBC)

The increase in the number of erythrocytes results in better oxygen transport, which results in improved fish health (Sattanathan et al., 2023). Also, the increase in the number of WBC in the groups treated with licorice and echinacea indicates that these plants, through their bioactive compounds, have immunostimulating effects. Kondera et al. (2021), also reported increased leukocyte counts in common carp fed thyme oil in their daily diet.

Cyprinus carpio fed for 56 days with 0.5-2% thyme, showed an increase in the number of leukocytes and a better resistance to fungal infections (ALsafah & AL-Faragi, 2017).

The mean corpuscular volume (MCV) varied between 181.65±1.88 μm^3 in A1 and 191.91±1.93 μm^3 in A3, the highest value being 203.80±4.83 μm^3 in AM (Figure 3).

Mean corpuscular haemoglobin (MCH) recorded values of 56.92±1.26 pg in AM and lower in fish given phytogenic compounds; 51.91±0.41 pg. in A2, 51.70±0.76 pg. in A3, respectively 51.16±0.44 pg in A1, the differences being significant ($p<0.05$) (Figure 4).

Mean corpuscular haemoglobin concentration (MCHC) was insignificantly increased in A1 (28.16±0.16) compared to control.

A significant decrease in this value was observed in the group fed with wild thyme, A3 (26.94±0.20) (Figure 5).

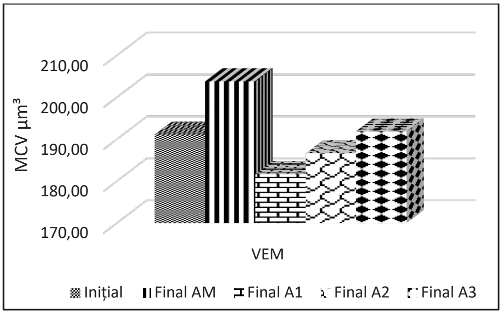

Figure 3. Variation of mean corpuscular volume (MCV)

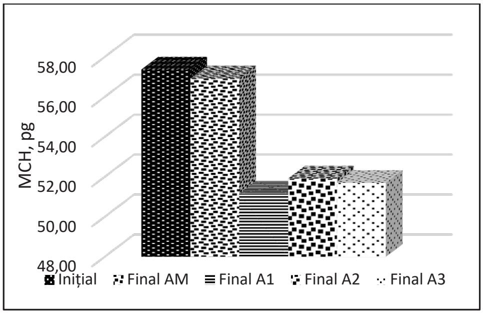

Figure 4. Variation of mean corpuscular hemoglobin (MCH)

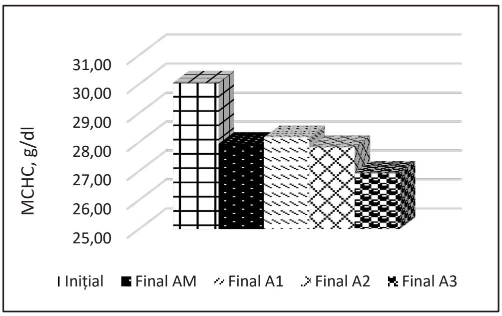

Figure 5. Variation of Mean corpuscular hemoglobin concentration (MCHC)

glucose in fish and can be a stress limiting factor (Bulfon et al., 2015).

Glucose values in this study were significantly lower in fish treated with the addition of licorice (91.14±0.02) and thyme (93.57±0.01) ($p<0.05$) compared to the control group (94.46 ±0.11) (Figure 6). The hypoglycemic effect of licorice glycyrrhizin is known, thus justifying the lower blood sugar values in the experimental lot A1 (Zhang et al., 2020).

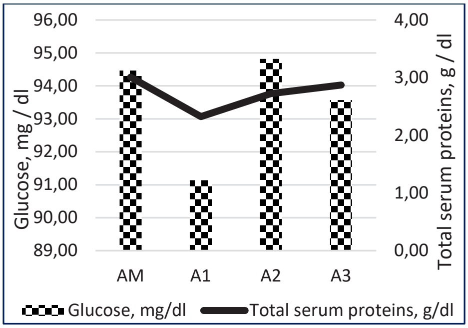

Figure 6. Evolution of glucose concentration and total serum proteins

Other authors have also reported decreases in blood glucose in fish treated with licorice. Sirakov et al. (2019) observed that the mean blood glucose values of rainbow trout fed licorice were 3.96% lower than those of the control group.

Total blood proteins were significantly lower in groups A1 (2.32 ± 0.02) and A2 (2.72 ± 0.01), while in group A3 (2.87 ± 0.05) the differences were insignificant (Figure 6).

These results were in contradiction with those of Sattanathan et al. (2023), who obtained an increase in the value of total serum proteins when a mixture of algae was included in the diet of *Labeo rohita*.

CONCLUSIONS

The results of our research generally indicate a positive response of hematological and biochemical indices to the supplementation of carp with various phytogenic compounds from licorice, echinacea and wild thyme. However, more research is needed to determine optimal concentrations of additives in other fish species.

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ZEOLITE FILTERS - TOOLS TO IMPROVE WATER QUALITY IN RECIRCULATING SYSTEMS IN AQUACULTURE

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Abstract

For the efficient and sustainable use of water in recirculating aquaculture systems, in order to improve water quality, zeolite filters have been used by retaining ammonia. The efficiency of the filters was tested in systems with volumes of 220 liters of water, populated with batches of 36 carp seedlings (Cyprinus carpio) in summer I. Horizontal filters with zeolite bed and composite filters, obtained from a granular mixture of silicate glass and zeolite, were used when filtering the water. For the determination of ammoniacal nitrogen, the continuous concentration criterion (CCC) and the maximum concentration criterion (CMC) were used. After 24 and 48 hours of water filtration using clinoptilolite filters, it was found that the maximum permissible values of ammonia in the water were not exceeded. Ammonia is absorbed in relatively large quantity, zeolite improves the filtration yield, which recommends the use of these types of filters in controlled recirculating systems in aquaculture.

Key words: *ammonia; continuous concentration criterion, clinoptilolite, maximum concentration criterion, organic recirculating systems.*

INTRODUCTION

At the World Conference on the Environment, held in 1992 in Rio de Janeiro, particular attention was paid to the concept of sustainability, which involves achieving a balance between economic growth and environmental protection as well as the use of alternative resources. Due to the current climatic conditions, it is necessary to use as rationally as possible the natural resources, especially the water. Aquaculture (FAO 2022) produced in 2020 49% of the total production of fish used for human consumption, i.e. 88 million tonnes, and this percentage is expected to increase in the coming years.

New technologies used in aquaculture ensure an increase in productivity. They must aim to protect the environment and the natural resource by reducing water and energy consumption and pollutant contaminants in the effluent waters of recirculating systems. Currently, more than 60 types of natural zeolites are known worldwide and more than 150 other types have been synthesized (Ghasemi et al., 2016). Natural zeolite bushes of very good quality are also found on the territory of our country. It is important to know and disseminate information on the efficiency of the use of zeolites in unconventional technologies (Mârza et al., 1991). Worldwide, it is tried to couple the adsorbent properties of the zeolitic - clinoptilolite volcanic tuff with various compounds (such as hydrotalcite – double hydroxycarbonate of magnesium and aluminum, which has anion exchanger properties) in order to obtain composites that have the property of retaining both cationic and anionic, inorganic or organic pollutants (Misăilă, 2004; Mishra & Jain, 2011). An economic and environmental advantage of the use of such filters is the low cost of adsorbent synthesis and the use of a matrix of natural origin that is not toxic and is widespread in nature. Zeolite volcanic bushes are an eco-alternative technological source of the future. They can be used as such, in membranes, in filters or in technological installations. Clinoptilolite zeolite has been registered in the European Community as a food additive DIN 53 770 since 2007 and declared safe for final consumers of meat, milk

or eggs from animals that have received zeolite in food or litter (Katsoulos et al., 2016; Pogurschi et al., 2017). A possibility to increase the mechanical strength and improve the absorption capacity of zeolites can be their incorporation into porous vitreous structures, with the obtaining of ecological zeolite-glass composites (Elisa et al., 2009; Sava et al., 2009; 2017).
The purpose of the work is to analyze the advantages of using filters based on clinoptilolite zeolite, as well as new filters, based on ecological clinoptilolite-glass zeolite composite in organic recirculating systems (SAR) populated with carp brood (*Cyprinus carpio*), especially with regard to the elimination by absorption of ammonia.

MATERIALS AND METHODS

The experimental researches, carried out in the Aquaculture Laboratory of the USAMV Bucharest, aimed at studying the influence of the natural clinoptilolite zeolite on the water quality and the development of the carp sapling (Cyprinus carpio) in summer I, from controlled systems. The duration of the experiment was 70 days. The controlled system used consisted of three aquariums with a capacity of 220 l each, the carp brood being divided into three batches of 36 individuals (Figure 1).

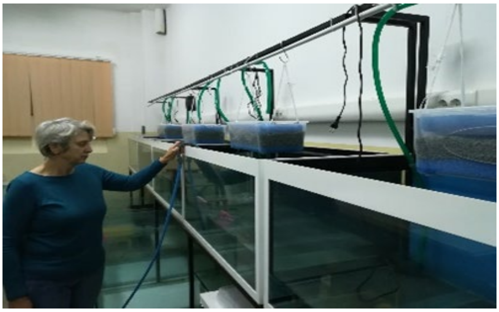

Figure 1. Controlled fish breeding system (original photo)

Clinoptilolite zeolite, used as a substitute for biological filters, is, from a chemical point of view, a natural aluminosilicate, with alkaline and alkaline-earth metals, crystalline and hydrated, which belongs to the group of tectosilicates (Emadi et al., 2001; Mansouri et al., 2013). The clinoptilolite chemical formula is:

$$(Ca, K, Na_2, Mg)_4Al_8Si_{40}O_{96}\ 24H_2O$$

From the mineralogical point of view, the zeolite used is of the clinoptilolite type. In its predominant chemical composition is silicon oxide (SiO_2) in the proportion of 68.75-71.30 followed by aluminum oxide (Al_2O_3) with a weight of maximum 13.10% (Table 1).

Table 1. The chemical and mineralogical composition of the clinoptilolite zeolite used (Zeolites Development data sheet)

Compound	Precent (%)
Chemical composition	
SiO_2	68.75-71.30
Al_2O_3	11.35-13.10
CaO	2.86-5.20
K_2O	3.17-3.40
Fe_2O_3	2.10-1.90
MgO	1.18-1.20
Na_2O	0.82-1.30
Calcination loss	9.77
Mineralogical composition	
Clinoptilolite	87-90
Plagioclase	2-5
Anherite	2-3
Cristobalite	4-5

According to the elemental chemical analysis (Figure 2), in the clinoptilolite zeolite the largest peaks belong to the main elements (silicon and aluminum), preceded by calcium, potassium, magnesium, iron, oxygen. From the category of microelements are distinguished titanium, zinc, zirconium, phosphorus and traces of sulfur, vanadium, chromium, copper and rubidium.

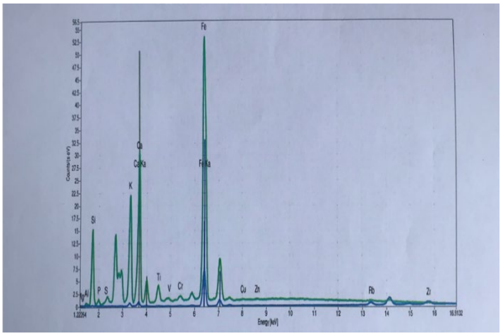

Figure 2. Chemical analysis of clinoptilolite zeolite powder (Zeolites Development data sheet)

The physico-chemical properties of clinoptilolite highlight the high porosity of zeolite, up to 44%, with a pore diameter of 0.4 nm, as well as the chemical resistance to the action of acids and bases (Table 2).

Table 2. Physico-chemical properties of clinoptilolite zeolite (Zeolites Development data sheet)

Property	Value
Softening temperature	1250°C
Melting temperature	1320°C
Flow temperature	1400°C
Appearance and smell	Gray-green, odorless
Porosity	32-44%
Pore diameter	0.4 nm
Mohs hardness	3.5-4
Water absorption	34-36%
pH	9
Density	2.377±0.002 g/cm^3
Chemical resistance	Resistant to the action of acids and bases
Thermal stability	up to 450^{0}C
Water solubility	Insoluble
Dangerous decomposition	Not applicable
Dangerous polymerization	Not applicable
Toxicity	Nontoxic
Total cation exchange capacity	175 meq/100 g

Water filtration was conducted using simple and composite zeolite filters (Sava et al., 2017). To make a simple filter, the amount of 4 kg of clinoptilolite was used, with the initial granulation of 3-5 mm, subsequently 2-3 mm. Zeolite was regenerated at 48 hours with saline (Ghasemi et al., 2016; Nicholas et al., 2017).
The filtration of residual solids from the water of recirculating systems for breeding carp juveniles was done before the water came into contact with zeolite, using sponges and filter cotton wool (Figure 3).

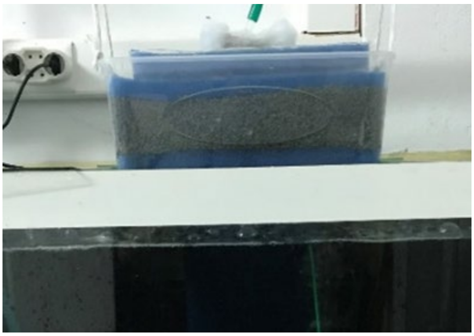

Figure 3. The filter with clinoptilolite zeolite (original photo)

For the realization of the composite filters sintered glass-clinoptilolite, called FSZ 1 and FSZ 2, glass with a silico-chalco-sodium composition was used (Table 3).

Table 3. Glass composition (Krystal Clear - Intex data sheet)

No.	Oxide	Percentage (%)
1	SiO_2	72.07
2	CaO	9.54
3	MgO	3.25
4	Na_2O	13.79

The glass was used in the form of granules with a maximum size of 0.50 mm and had a powder density of 1.6 x 10^3 kg/m^3 (Table 4).

Table 4. Glass properties (Krystal Clear - Intex data sheet)

No.	Property	Value
1	Absolute density	2.4 x 10^3 kg/m^3
2	Powder density	1.6 x 10^3 kg/m^3
3	Grains dimension	0.25-0.5 mm
4	Uniformity coefficient*	1.6

* According to (Radu and Ibriș, 2004)

The zeolite in the FSZ 1 composite filter had the finest grain below 0.125 mm, and the coarse one of 1.25 mm (Table 5).

Table 5. Particle size distribution of the zeolite used in composite filters

Particle size (mm)	Percentage (%)
0.5-1.25	6.24
0.25-0.50	12.39
0.125-0.25	1.01
Under 0.125	0.36
Total	20

In obtaining the FSZ 1 filter, a zeolite/glass ratio of 1: 4 and 20 g of zeolites and 80 g of glass was used. For pressing and binding of granules was used a solution of polyvinyl alcohol (PAVN) 8% in distilled water, in the proportion of 10%, relative to 100% granular glass-zeolite mixture. In this first variant, the composite filters with a diameter of about 20 mm were made in raw (Figure 4), by pressing with a hydraulic press, with a force of 2.5 Tf on the entire surface.

Figure 4. Composite filter - type 1 (FSZ 1) (original photos)

The pressed composite filter was inserted into an electric furnace with molybdenum disilicide bars Nabertherm - Germany. The thermal sintering treatment was aimed at obtaining a mechanical resistance high enough to cope with the passage of water. Even though the sintering temperature reached 600ºC, a porosity was retained large enough for this passage to be possible (Malherbe et al., 2006) (Table 6).

Table 6. Thermal treatment parameters for the obtaining of composite filter type 1 (FSZ1)

Temperature domain (ºC)	Duration (hours)
20-110	1
110	1
110-250	2
250	1
250-600	3
600	2
600-20	6
Total	16

The shrinkage of the diameter of the FSZ 1 filter after sintering is 1.4%, and the height contraction is 1.9%. The weight loss of the filter is 9.4% and the open porosity represents 49% of the total porosity (Table 7).

Table 7. Composite filter properties FSZ 1 type

Property	Value
Green diameter	20.24 mm
Sintered diameter	19.94 mm
Diameter contraction	1.4 %
Green height	17.41 mm
Sintered height	17.0 mm
Height contraction	1.9 %
Green mass	10.35 g
Sintered mass	9.38 g
Mass loss	9.4 %
Green volume	5.6 cm^3
Sintered volume	5.33 cm^3
Volume contraction	4.82 %
Green density	1.848 g/cm^3
Sintered density	1.759 g/cm^3
Absolute density *	2.36 g/cm^3
Theoretical volume	3.97 cm^3
Total porosity**	25.45 %
Open porosity***	12.47 %

The absolute density of the composite was calculated by a simple additive relationship:

$$d_c = d_s \times m_s + d_z \times m_z \quad (1)$$

where:
d_c = composite absolute density
d_s = glass absolute density
m_s = glass weight %
d_z = zeolite absolute density
m_z = zeolite weight %

The total porosity was calculated with the formula:

$$P_t = \frac{V_c - V_t}{V_c} \times 100 \quad (2)$$

where:
P_t = total porosity
V_c = sintered composite volume
V_t = theoretical volume

The open porosity was calculated with the formula:

$$P_d = \frac{P_t \times A_z}{P_z} \quad (3)$$

where:
P_d = open porosity
A_z = water absorption zeolite (Aquatech) = 16.21 %
P_z = total porosity zeolite (Aquatech)=33.08%.

In the second type of sintered composite filter, FSZ 2 (Figure 5) the proportions of glass and zeolite were preserved, and the grain of the zeolite used was between 0.5 and 1.25 mm. In this case, a 5 % PAVN solution was used in distilled water at the rate of 8% per 100% granular mixture of zeolite-glass.
The thermal sintering treatment was performed at a temperature of 620ºC, higher by 20ºC than in the case of the FSZ 1 filter (Table 8).

Figure 5. Composite filter - type 2 (FSZ 2)

Table 8. Thermal treatment of the composite filter type 2

Temperature domain (ºC)	Duration (hours)
20-110	1
110	1
110-250	2
250	1
250-620	3
620	2
620-20	6
Total	16

The shrinkage of the diameter of the FSZ 2 sintered filter is 12.08%, and of the height of 3.67%, higher than in the case of the FSZ 1 filter, but the weight loss is less than 7.66 g. The open porosity represents, as in the case of the FSZ 1 filter, 49% (Table 9).

Table 9. Composite filter properties FSZ 2 type

Property	Value
Green diameter	20.15 mm
Sintered diameter	19.73 mm
Diameter contraction	12.08 %
Green height	16.37 mm
Sintered height	15.77 mm
Height contraction	3.67 %
Green mass	9.41 g
Sintered mass	8.69 g
Mass loss	7.66 %
Green volume	5.22 cm^3
Sintered volume	4.82 cm^3
Volume contraction	7.66 %
Green density	1.804 g/cm^3
Sintered density	1.804 g/cm^3
Absolute density	2.36 g/cm^3
Theoretical volume	3.68 cm^3
Total porosity	23.57 %
Open porosity	11.55 %

Hanna Instruments' Aquaculture Photometer HI3303 and specific reagents from the same company were used to measure the water parameters, and to determine the temperature, thermometers for aquariums.

The continued concentration criterion (CCC) is the limit value of total ammonia resulting in unacceptable effects, i.e. more than 20 % decrease in survival, growth and/or reproduction (Eddy, 2005)

The maximum concentration criterion (CMC) is half of the final acute value of total ammonia (Eddy, 2005)

For the testing of the FSZ 1 and FSZ 2 filters, an assembly consisting of a PVC pipe was made inside which the filter was inserted, fixed with silicone. The filtration system was positioned vertically using a clamping system and through it was passed the amount of 100 ml of water coming from the aquarium and 100 ml of ammonia solution in distilled water, of different concentrations, between 1 and 2.3 mg/l.

Several consecutive crossings were made, after which the filters were regenerated with distilled water and reused, in order to follow the efficiency of zeolite regeneration.

RESULTS AND DISCUSSIONS

The initial use of zeolite with a grain size of 3-5 mm when filtering water from aquariums kept the ammonia values high. Although the regeneration of clinoptilolite was carried out at 24 hours, the ammonia values continued to increase slightly (Table 10). The water temperature was constant, of 22ºC, the pH value was between 7.5 and 7.6, and that of dissolved oxygen (DO) was 6.5-6.7.

Table 10. Water parameters

Aquarium	NH_4^+ (mgL^{-1})	NH_3 (mgL^{-1})	NH_3-N (mgL^{-1})
Initial	0.68	0.65	0.53
1	1.28	1.21	1.00
	1.48	1.39	1.15
	1.73	1.63	1.34
	1.89	1.79	1.47
Initial	0.70	0.66	0.54
2	1.22	1.15	0.94
	1.57	1.48	1.22
	1.66	1.56	1.29
	1.98	1.87	1.54
Initial	0.73	0.69	0.56
3	1.30	1.01	0.87
	1.84	1.74	1.43
	1.85	1.74	1.43
	1.99	1.88	1.55

By using the smaller-grained zeolite, respectively 2-3 mm, the values of ammoniacal compounds were lower after 24 hours, due to the smaller size of the zeolite particles (Table 11) (Asgharimoghadam et al., 2012).

The water samples were taken and analyzed 24 hours and 48 hours after the zeolite change.

Table 11. Water analysis after 24 and 48 ore of zeolite use

Aquarium	NH_4^+ ($mg L^{-1}$)	NH_3 ($mg L^{-1}$)	NH_3-N ($mg L^{-1}$)	NH_4^++NH_3 ($mg L^{-1}$)
1 Initial	1.29	1.20	1.01	2.49
(24 h)	1.76	1.66	1.37	3.42
(48 h)	2.99	2.82	2.32	5.81
(24 h)	1.16	1.09	0.90	2.25
(48 h)	3.30	3.11	2.56	6.41
(24 h)	1.21	1.15	0.94	2.36
(48 h)	3.25	3.07	2.52	6.32
(24 h)	1.54	1.45	1.09	2.99
(48 h)	2.68	2.53	2.08	5.21
(24 h)	0.98	0.92	0.73	1.90
(48 h)	2.62	2.47	2.04	5.09
(24 h)	0.82	0.78	0.64	1.60
(48 h)	2.18	2.06	1.69	4.24
2 Initial	1.47	1.38	1.13	2.85
(24 h)	1.86	1.75	1.44	3.61
(48 h)	3.22	3.04	2.50	6.26
(24 h)	0.76	0.72	0.59	1.48
(48 h)	3.42	3.23	2.66	6.65
(24 h)	1.13	1.06	0.87	2.19
(48 h)	3.43	3.24	2.66	6.67
(24 h)	0.92	0.87	0.72	1.79
(48 h)	2.39	2.26	1.86	4.65
(24 h)	0.82	0.77	0.64	1.59
(48 h)	2.92	2.80	2.30	5.72
(24 h)	0.96	0.91	0.75	1.87
3 Initial	1.37	1.29	1.05	2.66
(24 h)	1.83	1.72	1.42	3.55
(48 h)	2.97	2.80	2.30	5.77
(24 h)	0.64	0.61	0.50	1.25
(48 h)	2.78	2.63	2.16	5.41
(24 h)	0.85	0.80	0.66	1.65
(48 h)	3.18	3.01	2.47	6.19
(24 h)	1.48	1.39	1.15	2.87
(48 h)	3.53	3.33	2.74	6.86
(24 h)	0.95	0.90	0.74	1.85
(48 h)	3.19	3.01	2.40	6.20
(24 h)	0.92	0.87	0.71	1.79
(48 h)	2.39	2.26	1.86	4.65

The pH values of the water during the experiment were between 7.5-7.6, and those of dissolved oxygen between 6.5-6.7 mg/l, both parameters being within the normal permissible limits. The water temperature was 21-22°C.

Total ammonia values (NH_3 + NH_4^+) after 24 hours of filtration were between: 1.60 mg L^{-1} - 3.42 mg L^{-1} in the first aquarium; 1.59 mg L^{-1} - 3.61 mg L^{-1} in the second aquarium and 1.25 mg L^{-1} - 3.55 mg L^{-1} in the third aquarium.

The upper limit of total ammonia did not exceed the accepted limit of the continuous concentration criterion (CCC) which is 3.8 mg NH_3 + NH_4^+ mg L^{-1} (Eddy, 2005).

After the 48-hour filtration interval, the total ammonia values exceed the accepted limit of the continuous concentration criterion in all three aquariums, ranging from 3.42 mg L^{-1} to 6.86 mg L^{-1}.

The limit of the maximum concentration criterion (CMC) for the carp species is 8.4 mg NH_3 + NH_4^+ mg L^{-1} (Eddy, 2005).

The CMC criterion values were not exceeded either after 24 or after 48 hours of water purification using clinoptilolite zeolite (Ip et al., 2001).

Total ammonia nitrogen values, in all cases, after 24 and 48 hours, were well below the concentration of 15 mg L^{-1}, which is the limit for a 27 % decrease in survival rate (Farhangi et al., 2013).

Since CCC values after 48 hours have been increased, it is concluded that zeolite should be regenerated after 24 hours of continuous use.

In the fish organism, the decrease in ammonia toxicity can be achieved by decreasing its production, increasing the excretion of ammonia or converting ammonia into less toxic compounds intended for storage or excretion (Randall & Tsui, 2002). After exposure to higher ammonia values, plus endogenous ammonia, the organism of some species, including the common carp (Cyprinus carpio), can synthesize glutamine from glutamate and NH_4^+. Glutamine is stored in tissues and can subsequently be used as an oxidative substrate. The disadvantage lies in the high energy consumption of the body to perform detoxification (Eddy, 2005).

In the case of composite filters FSZ 1 and FSZ 2, the ammonia retention capacity testing was carried out by successive passages of either aquarium water or water and ammonia solutions.

Through the FSZ 1 filter, 10 passages of aquarium water with an NH_3 concentration of 1.57 mg/l were carried out at a temperature of 24°C.

The filter contains 1.87 g of zeolites and retained 62.42% of the ammonia in the test sample, i.e. 0.98 mg. One gram of clinoptilolite retained 0.52 mg of ammonia in a span of 4 hours and 39 minutes (Table 12).

Table 12. Water analysis after passing through the FSZ 1 filter

Parameter	Initial value	Final value	% absorption
pH	6.5	6.7	
NH_4^+	1.67 mg/l	0.62mg/l	62.87
NH_3	1.57 mg/l	0.59mg/l	62.42
NH_3-N	1.29 mg/l	0.48mg/l	62.79

The regeneration of the filter was conducted by washing with two liters of distilled water, to eliminate the retained ammonia. Through the regenerated filter, a new series of 5 passes of aquarium water with an NH_3 concentration of 1.85 mg/l was carried out. The total time interval was 5 hours and 15 minutes.

After regeneration, the FZS 1 filter retained only 60% ammonia from the test sample (Table 13).

Table 13. Water analysis after passing through the regenerated FSZ 1 filter

Parameter	Initial value	Final value	% absorption
pH	6.5	6.9	
NH_4^+	1.96 mg/l	0.78 mg/l	60.20
NH_3	1.85 mg/l	0.74 mg/l	60.00
NH_3-N	1.52 mg/l	0.61 mg/l	59.87

The filter was evaluated with aquarium water, which contained alongside ammonia, nitrites and nitrates, which reduced its ability to retain ammonia. Slower filtration and lower yield can be explained by plugging part of the pores of the sintered filter (Malherbe et al., 2006).

The FZS 2 composite filter was sintered at a temperature 20ºC higher than FSZ 1 in order to prevent the pores from plugging. For testing the filtering capacity of this type of filter, a laboratory-prepared ammoniacal solution was used. The aqueous ammonia solution with 2,07 mg NH_3/l was subjected to 10 successive filter passes.

Table 14. Ammoniacal solution after passing through the FSZ 2 filter

Parameter	Initial value	Final value	% absorption
pH	6.5	7.7	
NH_4^+	2.19 mg/l	0.08 mg/l	96.35
NH_3	2.07 mg/l	0.08 mg/l	96.14
NH_3-N	1.70 mg/l	0.06 mg/l	96.47

1.74 g of zeolite from the filter retained 96.14% of the ammonia present. During the experiment - 7 hours and 51 minutes, one gram of zeolite retained 1.14 mg of ammoniac (Table 14).

After regeneration, the filter was subjected to a new series of five passes of an ammonia solution with a concentration of 2.14 mg NH_3/l.

Following the second series of passes, which lasted 10 hours and 20 minutes, the 1.74 g of zeolite retained 97.66% of ammonia, which means that one gram of zeolite retained 1.20 mg of ammonia (Table 15).

Table 15. Analysis of the ammonia solution after passing through the regenerated FSZ 2 filter

Parameter	Initial value	Final value	% absorption
pH	6.5	7.7	
NH_4^+	2.27 mg/l	0.05 mg/l	97.8
NH_3	2.14 mg/l	0.05 mg/l	97.66
NH_3 - N	1.76 mg/l	0.04 mg/l	97.73

Although the sintering temperature was increased by 20ºC, from 600ºC to 620ºC, the filter showed the same tendency to increase filtration time after regeneration, which shows that either the filter pores clump after the first filtrations, or the open porosity decreases by potential chemical reactions inside the filter. It follows that, in future research, the cause of this decrease in open porosity will be precisely established, resulting in an increase in filtering time.

CONCLUSIONS

In the present study it was aimed at improving the quality of water in recirculating systems in aquaculture by using clinoptilolite zeolite. When using simple horizontal filters with a zeolitic bed, it was observed that in none of the cases presented were the values of the continuous concentration criterion (CCC) and the maximum concentration criterion (CMC) for ammoniacal nitrogen exceeded, both after 24 and after 48 hours of zeolite water purification. To improve the filtration efficiency, composite filters consisting of sintered glass and zeolite were made and tested. The FSZ 2 composite filter revealed an increased ability of zeolite to retain ammoniacal compounds, which means that the inclusion of clinoptilolite in glass does not lead to a decrease in this property. A problem that needs to be solved further is that both composite filters have filtered in increasingly longer times, which means that their open porosity has shrunk during filtering.

The clinoptilolite used to filter the water contributed to the maintenance of the medial conditions favorable to the growth and development of the fish from the controlled systems used, but also to ensuring a 100% survival rate.

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INNOVATIVE TECHNOLOGIES FOR FISH BREEDING WITH MINIMAL IMPACT ON THE ENVIRONMENT

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Abstract

This study refers to the common carp (Cyprinus carpio), which is an adaptable species that enriches the variability of quantitative and qualitative characteristics and increases genetic diversity. Local aquaculture populations of common carp, called "landraces," have developed due to different environmental conditions and breeding efforts. However, the introduction of carp in some areas has led to negative impacts on natural aquatic ecosystems. To improve the quality of economically important fish species, the variation in morphological, physiological, and biochemical characteristics is utilized. In this study, a patent application for a system for reproduction, selection, and growth of fish fry with the simulation of natural conditions is described. The article explains the method used to replicate the natural aquatic environment and create viable products with high genetic adaptability to its conditions. The process falls into the category of extensive aquaculture, promoting sustainable aquaculture by increasing the percentage of ecological and environmentally friendly productions. The study concludes with the results and the development of a set-up of the station for laboratory use.

***Key words**: aquaculture, artificial inteligence, future, technologies.*

INTRODUCTION

One of the most adaptable species in the wild and aquaculture conditions, the common carp enriches the variability of quantitative and qualitative characteristics and increases its genetic diversity.

Local aquaculture populations of common carp have developed within the species due to different environmental conditions, the efforts of fish farmers to breed them, and the relatively small size of the breeding population due to systems of strictly closed breeding grounds. Different genotypes were specifically developed after the middle of the last century and are referred to as "landraces" (Bakos, 1979). These populations are adapted to the local environment and have a high level of genetic diversity.

The introduction of *Cyprinus carpio* in many areas has led to a significant development of aquaculture, and carp farming now plays an important role in the economies of many countries. On the other hand, in some developed countries, such as the United States and Australia, where the species is not consumed outside the poorer segments of society, it is considered a pest (Dowal, 1996) and significant efforts have been made to eradicate it.

The negative impact of *Cyprinus carpio* on natural aquatic ecosystems is clearly observed in their behavior, such as uprooting and destroying aquatic plants as a result of their feeding habits (Laird, 1996). Carps also increase water turbidity by digging and mixing the top layer of the bottom, which decreases light penetration and destroys macrophyte populations in spawning grounds of photophilic species (Star, 2011; kerutokoi.com).

The main purpose of fish breeding is to improve existing breeds and hybrids and to develop new breeds, thereby increasing their productivity. To improve the quality of economically important fish species, their variation in many morphological, physiological, and biochemical characteristics is utilized. A significant proportion of this variation is heritable, and its value is very high in fish populations, which helps in the application of fish selection methods.

Compared to domestic animal husbandry, aquaculture is a relatively young science in

China and India, having been in vogue for a long time, but the domestication of fish and the creation of breeds that differ from their wild parents in terms of high productivity traits actually began only a few centuries ago. With the notable exception of the golden crucian carp, ornamental carp, and perhaps the common carp, few fish can be considered domesticated, even though some strains of trout, for example, are much more adapted to hatchery conditions than their wild counterparts (FAO, 1985).
Among the representatives of *Cyprinus carpio* suitable for aquaculture activity, only the common carp has been bred for a sufficiently long period, and distinct breeds of this species have been developed through selection. In the USSR, these include the Ukrainian carp, Ropsha carp, first-generation hybrids of domestic carp and Amur Wild carp, Nivchan carp, Central Russian carp, Kazakh carp, Kasnodar carp, Belarusian breed, and Parra breed (Kirpichnikov, 1981). In Israel, there is the carp "Dor-70" (Wohlfarth, 1980), and in Hungary, the Hungarian strain (Bakos, 1979).

MATERIALS AND METHODS

In order to carry out this work, the data that formed the basis of the patent application titled "System for Reproduction, Selection, and Growth of Fish Fry with the Simulation of Natural Conditions" was centralized. The patent is registered with the Romanian State Office for Inventions and Trademarks.

Materials

The block diagram of the installation for fish breeding and selection, as presented in Figure 1, describes the setup used inside the laboratory.

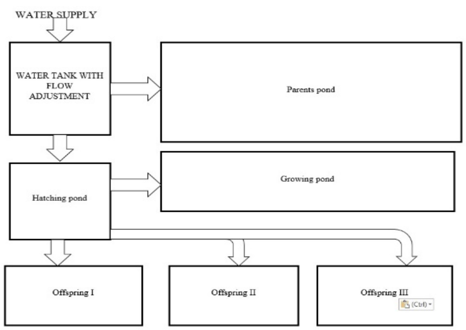

Figure 1. Block diagram of the installation for fish breeding and selection

The tanks are made of glass and filled with water from the breeders` aquatic environments.
A hydrological station is used to measure and monitor the qualitative parameters of the reference aquatic environment in order to replicate it in the reproduction station (Figure 2).

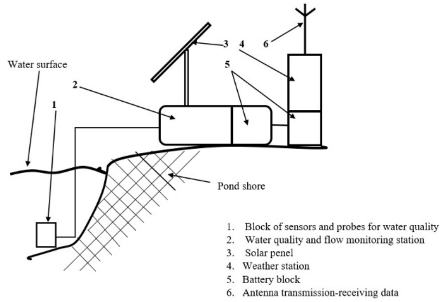

Figure 2. Installation for monitoring parameters of aquatic environments

Three specimens of *Cyprinus carpio* that have reached sexual maturity are represented by the biological material.

Methods

Portions of the aquatic environments from which the breeders come and where the offspring will be released are mapped out to be created inside the reproduction station (Figures 3, 4).

Figure 3. Overall map of fish farm for catch and release fishing (Google maps)

Figure 4. Map focusing on the area of interest with the exact area to be replicated clearly marked (Google maps)

All the materials used to replicate the natural aquatic environment are purchased from the reference aquatic environment, aiming to create viable products with high genetic adaptability to its conditions.

By applying these techniques, the process falls into the category of extensive aquaculture, at most semi-intensive, promoting sustainable aquaculture by increasing the percentage of ecological and environmentally friendly productions. It is especially important that the food is ecologically sound, with the possibility of carrying out a sustainable and efficient activity for the conservation of resources.

The installation for monitoring parameters of the aquatic environment has been installed on the shore of the lake that provides for the breeders.

By virtue of the application of new technology, namely the ability to multiply carp at any time of the year and to apply genetic analysis on the parents and offspring, electroanesthesia was used to anesthetize the adult specimens, both the female and the three males. (Șerban, 2020).

The applied method proved to be of real help, allowing the individuals to not be injured during manipulation, but it also led to the elimination of the semen without interfering with the pituitary gland. The observed semen was collected on a smear and analyzed in the physio-pathological analysis laboratory of USV Iași, with the result being the eligibility for reproduction, ascertaining the maturity and the ability to reproduce.

RESULTS AND DISCUSSIONS

After putting all the information together, we developed a set-up of the station shown in Figure 5 for laboratory use.

Figure 5. Laboratory set-up for hatching (original)

The selected biological material is represented by three *Cyprinus carpio* individuals: a female ornamental carp variety Koi Doitsu Kin Matsuba (Figure 6) and three male common carp varieties with scales, mirror, and topless (Figure 7.).

Figure 6. Koi Doitsu Kin Matsuba under laboratory conditions (original)

Figure 7. Aquaculture carp, var. mirror under laboratory conditions (origanl)

The ornamental carp variety Koi Doitsu Kin Matsuba comes from a controlled environment, with special feed being administered during the growth process to maintain the color and exceed the standard growth parameters, an important aspect in aquaculture (Bhaskar, 2015).

Feeding was carried out four times a day during the periods when the water temperature was between 18-28°C, and gradually, depending on the evolution of the temperature, a single ration per week was applied with the commercial feed, which falls qualitatively into the premium class for ornamental fish. The qualities that recommended this feed were the presentation in floating extruded form, with 4mm granulation, and the nutritional composition of 37% protein and 8.5% fat.

The female Koi Doitsu Kin Matsuba was 13 months old, 25 cm, and 900 g at the time of purchase and transfer to the laboratory. It was in perfect health. For the last 5 months, it has been kept in a glass aquarium with 130 l of water from its place of origin. It has been fed the same food as before acquisition, and only aeration and filtration operations have been applied to the aquatic environment. Filtration was applied during the summer, and now, with the aquatic environment temperature at 8-9°C, filtration is no longer necessary and feeding is applied once every 7 days. After 5 months in the laboratory, the specimen's metric indices are 31 cm and 1600 g, which falls within the limits of growth rates of ornamental carp (Table 1).

Table 1 Growth rate of ornamental carp*

Age (years)	Total length (m)	Body mass (kg)
0.5	0.12	0.05
1	0.23	0.3
1.5	0.32	1.1
2	0.39	1.4
2.5	0.45	2.25
3	0.51	3.15
3.5	0.55	4
4	0.59	4.95
4.5	0.62	5.7
5	0.64	6.55
5.5	0.67	7.3
6	0.69	7.86
6.5	0.71	8.48
7	0.72	9.03
8	0.73	9.5
9	0.75	10
10	0.76	10.4

*Adapted after koi owner`s notes (original)

The specimen purchased for the present study comes from a group of fifty ornamental carp of various varieties, all of the same age, purchased in Hungary. During its development, from the age of three months, it was kept in an outdoor pool on EPDM sheeting (Ethylene-Propylene-Diene-Monomer mixed with carbon black, oils, vulcanizing agents, and other auxiliaries). Great importance was given to transparency in order to observe the reactions of the carp and to identify any possible dysfunctions in the new aquatic environment.

Regarding stress, the female did not show extreme forms of manifestation, like the specimens of common carp that were brought to the laboratory. Used to the high transparency of the aquatic environment, it had no specific reactions to stress after transport or during handling during the study (e.g. no jumps when cleaning the filter).

The males selected for the present study come from an extensive rearing system, wherein feeding is almost non-existent, and food is provided by the natural productivity of the accumulation into which they are released in autumn, after being harvested from the pre-development ponds. This system of growth lends itself to accumulations with very large surface areas of water, where it is not profitable to invest in feed or incentives for the natural productivity of the pond.

Under these conditions, the fish are closer to the category of wild ones, exhibiting some characteristics in this regard. Carp raised in an extensive system differ from the others by the orange color of the lower part of the body, high tonicity, and vigorous appearance. At the time of harvesting, they are manifested by strong jumps and kicks that have a prolonged duration and a high intensity compared to common carp intensively raised, for example. Thus, due to this behavior and their vigor, they only lasted a maximum of 72 hours in the laboratory. Due to the strong impact on the walls of the aquarium, they presented parameters incompatible with life within a maximum of 3 days.

The three males selected to be transported to the laboratory - the scaled, mirrored, and topless varieties - were bred in June 2021 and are currently "parked" in a rearing pond at the collaborating farm. To ensure that there is no risk of losing the parents, they are kept in the conditions of the farm. They will only be brought to the laboratory for the application of reproductive procedures and the collection of samples for genetic analysis.

Carp length and body mass are parameters of interest in aquaculture, and in the extensive rearing system, the nutritional quality of the final product tends to be as close as possible to that of wild carp. As a result, Tables 2 and 3 highlight the standards encountered in wild carp and the limits of the recommended parameters in aquaculture, respectively.

Table 2 Growth rate of wild carp*

Age (years)	Total length (m)	Body mass (kg)
1	0.15	0.088
2	0.22	0.286
3	0.29	0.640
4	0.37	1.190
5	0.43	1.893
6	0.48	2.495
7	0.52	3.192
8	0.57	4.108
9	0.61	5.156
10	0.65	6.042

*after Papadopol, cited by Kaszoni, 1974

Table 3 Development periods and body mass of aquaculture common carp*

Period	Duration	Body mass (g)
Larval	3-7 days	0.025-0.05
Juvenile	15-30 days	0.2-1.0
Fingerlings	45-85 days	25-50
Youth	120-170 days	250-500
Adult	120-170 days	>1 000
Breeder	2-4 years	>4000
	1-2 years	>3000
	0.75-1 years	>2000

*According to FAO, 2022 and Bud - adapted

By comparison, it is easily seen that wild specimens weigh more than 1,000 g at the age of four years, while in aquaculture systems this weight is reached much earlier, after the second summer, at 16-17 months.

Specimens considered eligible for the present study fall within the standard measurements recommended in aquaculture: 27 cm and 1500 g (with scales), 27 cm and 1450 g (mirror), and 28 cm and 1500 g (topless). The three males are part of the same batch, and the same development technologies were applied to them; the differences are only registered as an aspect of their own evolution in the extensive system.

The semen obtained from the parents is divided into three equal parts and evenly distributed in the three aquatic environments that are the subject of the study: the aquatic environment of males, females, and bottled water.

These environments are in three aquariums, with each aquarium being introduced to 150 liters of water from each environment.

To create the necessary conditions in nature, *Pinus sylvestris* branches are placed as evenly as possible to cover as much of the available surface as possible, providing support for the adhesion of the fertilized eggs so that they do not pass underneath (Figure 8.).

Gauze "baskets" were created inside each aquarium and attached to the edges of the aquarium with special glass hooks in order to achieve aeration, swirling, and filtration inside the aquatic environment without disturbing the spawn attached to the pine branches.

Aeration (Figure 9), filtering, and heating (Figure 10) operations were applied to each environment. As seen in Figure 11, the aerators used had adjustable flow and direction, making it possible to swirl the water under the gauze "basket". On the opposite side, the filters were mounted, which also had the aeration function. Thus, the aquatic environment was swirled in both directions, ensuring the necessary conditions for the development of the larvae. On the same side as the filter was positioned the thermometer, which had an adjustment for the temperature value. By swirling the water on the opposite walls of the aquarium, the temperature was kept constant throughout the mass of the aquatic environment, avoiding large temperature fluctuations that are very dangerous for the development of future offspring.

Figure 8. Set-up for fertilized spawn (original)

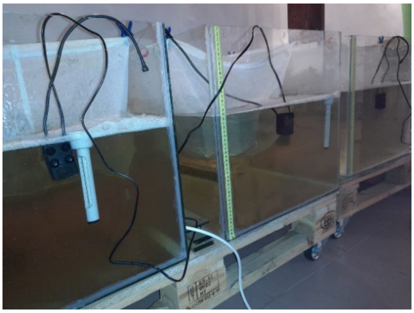

Figure 9. Aeration method applied to aquariums (original)

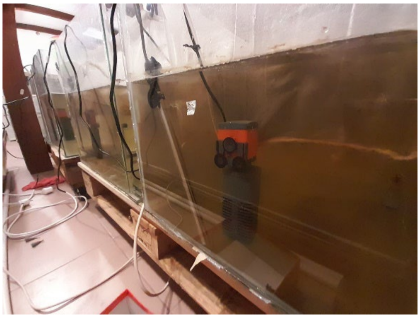

Figure 10. Method of filtering and heating aquariums (original)

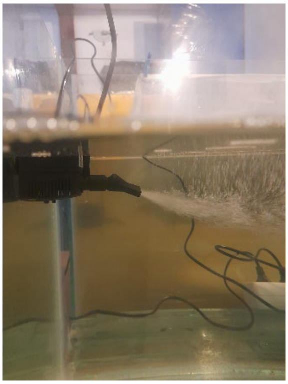

Figure 11. Aerator in operation (original)

To collect the data and create the database, a program specially developed for the used installation was used. It allows the user to set the time period during which the recordings are made, as well as the minimum and maximum limits of the monitored parameters, and it issues warnings regarding the recorded fluctuations (Figure 12.).

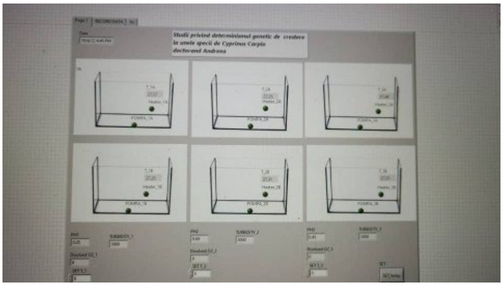

Figure 12. Software used in data collection (original)

After collecting the data, a sufficient database was created to conclude the effects of the techniques and technologies applied in the study at the aquaculture laboratory level.

CONCLUSIONS

The study describes the development of an installation for the reproduction, selection, and growth of fish fry with the simulation of natural conditions. The installation uses a hydrological station to monitor and replicate the qualitative parameters of the reference aquatic environment and biological material from three *Cyprinus carpio* individuals. The female is an ornamental carp variety Koi Doitsu Kin Matsuba, and the three males are common carp varieties. The feeding is carried out with a special feed, with high nutritional value, and is applied gradually, depending on the temperature of the water. Electroanesthesia was used to anesthetize the adult specimens, both the female and males, and the observed semen was collected on a smear and analyzed for eligibility for reproduction. The results showed that the setup can successfully replicate natural conditions for fish breeding, selection, and growth, promoting sustainable and environmentally friendly productions. The study's implication is that the findings can contribute to sustainable aquaculture and the conservation of resources. Further research is needed to explore the potential of the installation and the possibilities of genetic analysis.

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WILD BOAR SURVEILLANCE THROUGH ELECTRONIC MODULE "HUNT" MOBILE APPLICATION IN BULGARIA

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Abstract

African swine fever is a disease that affects representatives from Suidae family and leads to serious economic losses and ecological damage to swine population. A major role in the etiology of the disease is played by wild boars, both as a vector and as a reservoir. Due to this fact, monitoring the spread of the disease among feral pigs is one of the main factors for the prevention of the disease. For this reason, in 2019, the Bulgarian Food Safety Agency launched `Module "Hunt" application. The application allows hunters both easy and quick sending of data on the sample taken from a shot or found dead animal, as well as checking the result just by writing the sample number, while location, date and time of taking the sample are obtained automatically by the application. The current study analyzed both application performance (input, output of data) and data on number of samples taken from hunted feral pigs, percentage positive results from shot animals, proportion of samples taken from wild boars found dead with ratio of positive samples from them.

Key words: *African swine fever, surveillance, wild boars.*

INTRODUCTION

Due to the huge economic importance of the African swine fever disease, the fight for prevention or eradication of the disease is a priority in the agricultural policies of the countries. Wild boars play a major role in the spread of the disease. They are involved both directly, through contact, in low biosecurity of pig farms, and indirectly, e.g. through contaminated feed or food waste (Yoo et al., 2019; Schulz et al., 2021). In addition, wild boars play the role of a reservoir for the disease (Gervasi et al., 2019; Dixon et al., 2020). This makes it impossible to control ASF without controlling the infection in feral pigs. Disease surveillance is one of the main methods of control. Countries affected by the disease or those at risk of its introduction take different measures to control the disease as far as feral pigs are concerned.

In Italy, where the disease is already present, regarding passive surveillance in wild boars, everyone is required to report discoveries of carcasses found in the field, and the local veterinary service collects samples from these animals (including wild boars killed in motor vehicle accidents). Wild boar sampling was planned in order to rule out ASF virus infection in the general wild boar population. The ASF plan includes sampling of all wild boars found dead (Iscaro et al., 2022).

To prevent the disease from entering its territory, following an outbreak of the disease in Belgium near to the border, France is introducing three new protocols for the active search for dead wild boars in the border area, which complement the standard surveillance in level III risk areas: patrols by volunteer hunters, professional systematic search of the area and use of dogs. These protocols complement each other in terms of location and time. The main objectives of the designed surveillance system are to ensure early detection in the event of disease introduction and to support the area's continued free status (Desvaux et al., 2021).

On the other hand, as mentioned above, one of the main routes for the spread of the disease in both wild and domestic animals is the remains of contaminated food. For this reason, it is vitally important that meat from infected animals is not allowed for consumption or food preparation. This applies to the greatest extent to the meat obtained from wild pigs, and the timely detection of infected meat from the shot animals is of great importance to prevent the spread of the disease (Guberti et al., 2022; Dixon et al., 2020; Mazur-Panasiuk et al., 2019).

In order to performs qualitative and timely control, it is important that the information on the found carcasses of wild pigs, the data on animals shot, the patho-anatomical findings and data on the samples taken reach the competent authorities and laboratories as quickly and easily as possible. Getting a result quickly also plays an important role in the disease surveillance process. The use of database-related mobile applications, in addition to fast and easy data transfer, provide a volume of information that can be used both for various analyses and for visualization of the received data (Beyene et al., 2018; Moses et al., 2021).

For thousands of years, maps have been used for display and analysis of geographic information. We can use the visualization of sample results on a map for visualization, retrieval and analysis of spatial data. Geographic information systems (GIS) in animal control are mainly used for outbreaks of notifiable animal diseases. They support the veterinary officer in defining restriction areas, assessing the number of animal holdings and animals and planning control measures. GIS makes routine tasks easier, e.g. by enabling the establishment of sampling plans or herd statistics for a specified area.

Geographic analytical methods help to investigate the spatial and temporal spread of animal diseases and to describe the risks. The results of such studies can be used as a basis for spatial simulation models which describe the spatial spread of the disease (GIS: Friedrich-Loeffler-Institut, n.d.; Norstrom et al., 2001; Tadesse & Amare, 2021). GIS have been widely used by researchers during the Covid-19 pandemic (Elsheikh, 2022; Kabir et al., 2021).

In September 2002, the Institute of Epidemiology (Friedrich-Loeffler-Institut) started the development of an animal based database system on the epidemiological situation of swine fever in wild boar in Belgium, France, Germany (federal states of North Rhine-Westphalia, Rhineland-Palatinate, and Saarland), Luxembourg and the Netherlands. This project was carried out in close cooperation with the working group for swine fever in wild boar of the European Commission. The database was realised as an internet based project and permits data entry and analysis via an internet browser (EURL CSF/ASF Database: Friedrich-Loeffler-Institut, n.d.).

After the entry of ASF in Bulgaria in 2018 and the sharp increase of cases in 2019, by order of the executive director of the Bulgarian Food Safety Agency the mobile application Module “Hunt” was launched on 10.07.2019.

MATERIALS AND METHODS

Mobile application. Module "Hunt" is an application for mobile phones that aims to facilitate the process of registering and sending samples from game, allowing the registration of a sample directly through the application, eliminating the paper copies of a letter to the laboratory. The application automatically reports the GPS coordinates of the place where the sample was taken, or where the animal was shot. The date and time of sampling are automatically recorded. The data are saved on a server. ASF testing laboratories can access these data and the result is re-entered electronically and can be accessed through both the mobile application and web-based application.

Data. In the application, in addition to the automatically generated data, data on the type of animals are entered (due to the fact that additional functionalities have been added to the application to control of a different wild animal diseases). After choosing the type of animal - wild boar, the data that must be entered are:

Sex of the animal

Shot or found dead. If the animal was shot, information is filled in, whether the animal was visibly healthy or with atypical behaviour. If an animal carcass is found, information is provided as to whether the carcass is fresh or in the process of decomposition.

Information on the age of the animal. Offspring - up to one year, one to two years, young animal - 2-3 years, and animal over 3 years.

Weight - up to 50 kg, 50 to 100 kg, over 100 kg.

For which disease the sample will be tested. There have to be selected for which disease the sent material should be examined, as well as the type of material.

Unique sample number - barcode.

Attached photos. The application also allows the introduction of photographs, in the case of patho-anatomical changes that would be important in establishing the diagnosis.

Samples. Data can be entered into the application for both shot and found dead animals. According to the sampling instruction, samples are taken from every wild boar shot or found dead. An organ sample from the spleen and a blood sample are taken from the shot animals. The same samples are taken from an open fresh carcass. A tubular bone is taken from corpses in stages of decomposition or from the remaining skeleton of an animal and sent for examination.

Organization for sending the samples: Each hunting group or every hunter who would send samples has barcodes, two by two with the same unique number provided by the hunting association of which he is a member. The samples taken from the shot wild boar are placed in a vial on which one of the barcodes is affixed, the second is affixed to the carcass, which is not allowed to be used until a negative result is obtained. The samples are sent to the laboratory by the hunting company, and the laboratory accesses the available data related to the sample by dialling the corresponding barcode in the application. A study protocol is re-entered into the application, and for the result, database can be accessed through both a mobile and a web-based application.

Data. In addition to the specific results for a given sample, the application creates a database of collected samples from its inception to the present date. The application has a public part where anyone interested can choose data on the type of game and disease for which he/she wants to receive data, the period of sending the samples (start and end date), the territory where the sample was taken (the whole country, a specific area, or municipality from the district). The data is received in tabular form and visualized on a map. When the mouse pointer is placed on the point visualizing a certain sample, the data for the corresponding sample is loaded on the screen. The differences in the colour with which the sampling locations are marked (negative - green, positive - red) enables quick orientation.

The official veterinarians' individual access, which is protected by a username and password, enables a more detailed review of the information.

RESULTS AND DISCUSSIONS

For the period from its commissioning to 31.12.2022, 64,175 samples have been entered into the application for 42 months. From all these, 2,676 were positive, 61,347 were negative, and unfit and blocked were 152 pcs. For comparison, in Estonia, 62,944 data records were available for 84 months of study, of which 60,238 originated from active surveillance (from a hunted wild boar) and 2,706 from passive surveillance (from a wild boar found dead, shot, sick or involved in a traffic accident accident). For Latvia, 102,321 data records were analysed over 83 study months. Of the samples analysed, 99,665 were from active and 2,656 from passive surveillance. Lithuanian data are available for 72 months of study. A total of 87,307 data records were analysed, of which 83,566 data came from active and 3741 from passive surveillance. The data for all three countries originate from the European Union wild boar CSF/ASF surveillance database (https://surv-wildboar.eu) (Schulz et al., 2022).

The data required to enter a sample in the Module "Hunt" correspond to a large extent to the data described in the Data collection chapter in African swine fever in wild boar ecology and biosecurity - second edition FAO Animal Production and Health Manual No. 28 (Guberti et al., 2022). According to this manual, the aim of data collection is to improve understanding of animal diseases and the capacity to control and eradicate them. Data collection and analyses are an essential part of any animal disease surveillance programme, acting as a useful tool to measure the efficacy of control and eradication strategies, and eventually to highlight weak points. A sample collection form has been created that includes the basic data to be collected. In addition to the basic data, it is important to include the latitude and longitude of the location where the animal was shot or found dead. According to the cited guide, geographic data are suitable for studying the spatial and temporal evolution of infection. Latitude and longitude are easy to register using a basic smartphone. In affected hunting grounds may have hunting lodges with specific coordinates and thus used as a proxy for the location. A dedicated mobile application can be a very useful solution, facilitating the reporting process by hunters when it comes to collecting samples from hunted animals or carcass finds (Guberti et al., 2022).

Similar data collection was obtained in many surveillance programs. The relevant authorities approved the use of the data. Each data record corresponds to the surveillance data for a single wild boar and contains information about place (county, municipality and the smallest administrative unit, where the animal was found or shot), the time (day/month/year) of sampling, age, sex of the wild boar and the origin of sample. The "origin" refers to how it was recorded: whether the sample was taken from an apparently healthy hunted wild boar (active surveillance) or from a wild boar found dead, shot because it was sick or killed in a road traffic accident (passive surveillance). Furthermore, the data of the laboratory test results were recorded (Schulz et al., 2021; Dellicour et al., 2020).

Data entry into The European Union Wild Boar Surveillance Database (https://surv-wildboar.eu), includes characteristics of the individual wild boar (sex, age, cause of death and location) as well as the results of laboratory diagnostic tests (virology and/or serology) (EURL CSF/ASF Database: Friedrich-Loeffler-Institut, n.d.).

Data entry in the Module "Hunt" application enables any interested person to enter data, the only required access information being a mobile phone number. The data can also be obtained through the application downloaded to a mobile device or through a web browser. The publicity of the data at the Module "Hunt" is of a great importance so that the citizens and mainly interested parties can get a clear idea of the extent of the spread of the disease. The individual account provides detailed information on each criteria set when entering the sample as well as the results entered by the laboratory. Access to EU CSF/ASF database is restricted by safety mechanisms. The users must authenticate themselves by user names and passwords (EURL CSF/ASF Database: Friedrich-Loeffler-Institut, n.d.).

Recording the automatic receipt of the coordinates where the sample was taken and the time and date of collection, allows the veterinary authorities to take immediate measures in case of a positive result of the sample. Zones can easily be defined, additional measures can be taken if necessary. The intensity of distribution in a relevant area can be estimated. The visualization of the map enables a clearer idea of the distribution and intensity of the disease in a specific area, creating geographic models, detailed information for disease forecasting, epidemic forecasting, identification of disease clusters or hotspots, creation of buffer zones, and for evaluating various strategies to prevent the spread of infectious diseases (Tadesse & Amare, 2021; Forth et al., 2023).

The Module "Hunt" visualizes the test results of the samples with different colours, which clearly shows the results by region. The results of all samples can be visualized (Figure 1), or specific results - positive (Figure 2), negative, unsuitable, blocked. The application provides the possibility to filter the samples by administrative region - district or municipality, as well as introducing a filter for the time period of sending the samples. These options are valid for both the public version of the application and the one accessible by veterinary authorities with authorization.

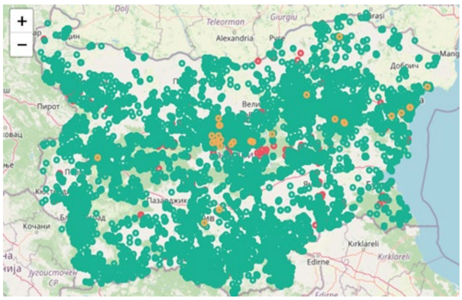

Figure 1. Visualisation of all samples recorded in Module "Hunt" for the period 2019-2022 (public account access)

Figure 2. Visualisation of all positive samples recorded in Module "Hunt" for the period 2019-2022 (public account access)

The European Union database also shows in addition to the analysis in the form of a table that can be filtered by time period, administrative units (e.g. Member State, Federal State, Region), age classes and results, the Internet map server also allows to display of the results in the form of a map according to administrative boundaries and topographic features (hunting module does not provide topographic features). Map Explorer connects the laboratory results with the corresponding areas of restriction and vaccination and facilitates the coloring and display of the maps. (EURL CSF/ASF Database: Friedrich-Loeffler-Institute, n.d.).
The possibility of obtaining data through active and passive surveillance leads to obtaining the most accurate information about the spread of the disease, as well as increases the percentage of found positive cases. In the Module "Hunt" the results can be obtained not only for the whole country but also for individual regions. Figure 3 shows the distribution of positive results in the 28 administrative regions of the country, for the period from the entry into operation of the application until the end of 2022. The diagram confirms the data, that although unevenly distributed due to the geographical and natural features of the regions, wild boar disease occurs in all regions of the country.

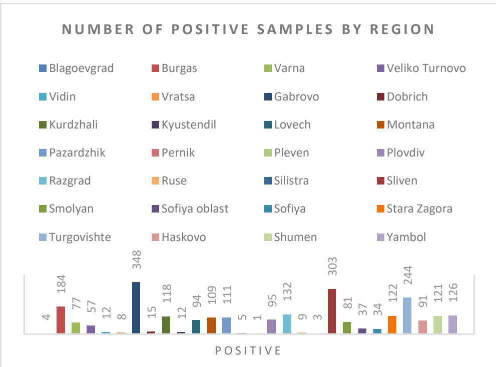

Figure 3. Number of positive samples by administrative region in Bulgaria

The ability to visualize the results allows a visual model of the spread of the disease to be created. (Depner et al., 2017; Dellicour et al., 2020).

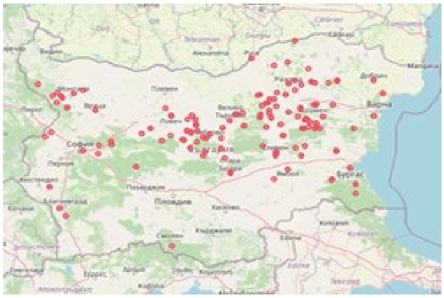

Figure 4. Visualization of positive samples of ASF in Wild boar – 2019

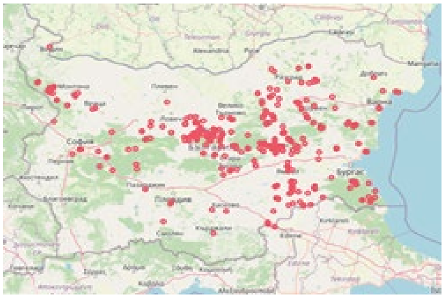

Figure 5. Visualization of positive samples of ASF in Wild boar – 2020

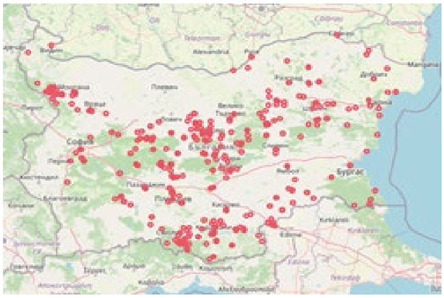

Figure 6. Visualization of positive samples of ASF in Wild boar – 2021

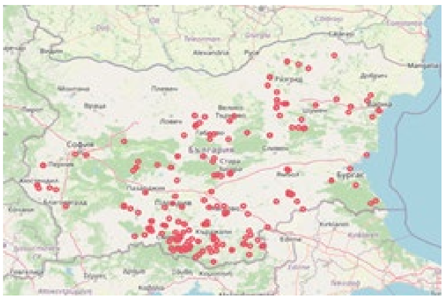

Figure 7. Visualization of positive samples of ASF in Wild boar - 2022

From the images above, we can get a clear idea of the territorial distribution of ASF in Wild boar for the research period 2019-2022. The number of positive samples had increased in territories unaffected or slightly affected by the disease, and decreased in territories where the incidence was high. The long-term retention of the amount of positive samples in the central part of the country is due to a huge mountain massif in terms of territory - Stara Planina mountain, creating conditions, on the one hand, for maintaining a high population of wild boars, and on the other hand, hindering the movement of animals over such large distances as it is possible to pass in the plains and foothills. All this makes the dynamics of the disease smoother, by keeping a relatively high percentage of morbidity for a longer time.
From the analysis of the data obtained by the application (Figure 8), it is possible to track the dynamics and intensity of the spread of the disease. From the data below it is clear that the percentage of positive, out of the total number of examined suitable samples among the population of wild boars, increased sharply and reached its peak in the year after the disease entered the territory of the country. The number of samples also increased, over the next two years the percentage of positive samples to all sent gradually decreased.

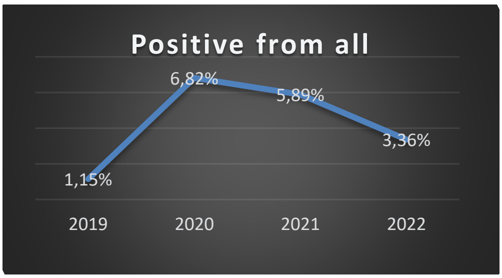

Figure 8. Percentage positive from all samples - 2019-2022 period

Similar trends are observed in other studies. The larger number of samples tested at the beginning of the outbreak is likely due to several factors. It can be assumed that the motivation and hope of successfully eliminating the disease led to increased efforts to hunt or find and sample wild boar. In addition, animal population density was significantly higher shortly after the introduction of ASF than in subsequent years, so more wild boar were available for sampling. A decrease in the number of samples obtained from dead wild boar has been described as an indication of a late phase of the epidemic (Schulz et al., 2022).
The same dynamics are observed in the percentage of samples of dead animals found, out of the total number of samples sent (Figure 9). From this it can be concluded that, following the peak of the disease and the peak of mortality from ASF in wild boars, it was also normal in the second year of the disease, after which it gradually decreased. We consider that the data analyzed do not give 100% credibility, due to the influence of other factors - the downturn from Covid 19, etc.

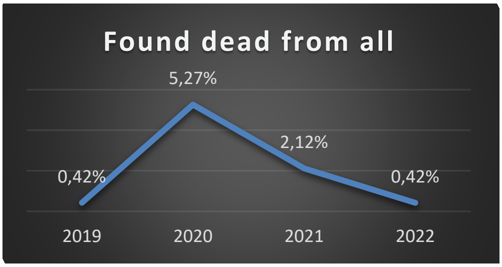

Figure 9. Percentage samples of found dead animals, from all samples - 2019-2022 period

Knowledge of this percentage is important because wild boars found dead represent a main sign of alert, especially when they are found in clusters (Ho et al., 2022).
An indicator of the intensity of the disease can also be the percentage of positive results from samples of dead animals found. According to the analysis of application data, the trends in positive results from dead animals found were the same as for the total number of results and mortality. A sharp increase in the number of positives in the second year after the infection entered the territory of the country and a gradual decrease in the following two years were detected. Unlike positive reactions from all animals which reach close to 7% at their peak, here it reaches 94% positive reactions from the sent samples (Figure 10). This can be considered as an indicator of the intensity of disease in feral pigs.

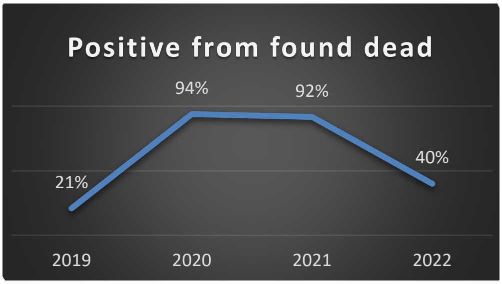

Figure 10. Percentage samples of found dead animals, from all samples - 2019-2022 period

In Lithuania, the average prevalence of ASFV-positive wild boar found dead, as determined by PCR, was 65.7%, while the serological prevalence in hunted animals (active surveillance) was only 0.45%.(Sauter-Louis et al., 2021).

When presenting the analysis results of the Module "Hunt" application, we cannot fail to mention one of its main functions - easy and quick sending of samples and receiving a result. Working with the application in its part of sending sample data and receiving the result is easy and intuitive. Sending samples from shot animals not only constitutes surveillance of the disease and tracks its spread, but by marking the sample and the carcass of the killed animal with barcodes with an identical number, it plays an important role in measures to prevent the spread of disease through animal products. The meat is released for consumption only after receiving a negative result through the application. In the case of a positive result, the carcass of the animal, according to EU legislation Regulation (EC) No. 1069/2009 of the European Parliament and of the Council of October 21, 2009, art. 8 point a(v), whole bodies and all parts of the body, including treated and untreated skins of wild animals suspected of being infected with a disease that is communicable to humans or animals are Category 1 material and are buried under biosecurity measures preventing the spread of the disease.

The advantages of working with the application described above are also applicable when working with found carcasses of dead animals. It not only makes it easier for hunters to send the samples, but because the data can be sent quickly and easily, it increases the percentage of sent samples from dead animals found. The effective and safe disposal of infected carcasses of dead animals plays a crucial part the disease control, because of their role in disease epidemiology (Guberti et al., 2022; Schulz et al., 2021).

CONCLUSIONS

Incorporating the data recommended by the FAO Manual to unify the information needed for disease screening application facilitates the analysis and sharing of data with other countries. The possibility of territorial and temporal filtering of the data allows the preparation of detailed analyses.

The geographic model allows easy tracking of the dynamics of the disease, its spatial movement and intensity. It facilitates delineation of areas and creation of geographic distribution models and simulations.

Making the data public allows the wide public and stakeholders to gain insight into the intensity and geographic distribution of the disease.

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ECOLOGOHELMINTHOLOGICAL INVESTIGATION OF *Cobitis elongata*, *Cobitis taenia*, AND *Sabanejewia bulgarica* (Cobitidae) FROM THE DANUBE RIVER, BULGARIA

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Abstract

For the period 2019-2021, three fish species of the family Cobitidae, caught from the upper section of the Danube River in Bulgaria, were subjected to ecologohelminthological investigation. Four specimens of Cobitis elongata Heckel & Kner, 1858 (two specimens from Kudelin biotope and two from Koshava biotope); six specimens of spined loach (Cobitis taenia Linnaeus, 1758) (from Koshava biotope) and one specimen of Sabanejewia bulgarica Drensky, 1928 (from Kudelin biotope) are objects of research. The trematode Asymphylodora tincae (Modeer, 1790) Lühe, 1909 was reported for the first time as a helminth of S. bulgarica. The nematode Pseudocapillaria tomentosa (Dujardin, 1845) Moravec, 1987 was reported for the first time as a helminth of C. elongata. Kudelin and Koshava biotopes are new habitats for the found helminth species. During the helminthological examination of C. taenia, infection with helminths was not found. The study provides new data on helminth fauna and ecological indices (MI, MA, and P%) in the helminth communities of C. elongata and S. bulgarica.

***Key words**: ecological indices, fish species, helminth species, Koshava, Kudelin.*

INTRODUCTION

The Danube River is the second longest river in Europe. The river is distinguished by an exceptional diversity of ichthyofauna (Juhásová et al., 2019). Studies on the parasite fauna of fish from the Danube River and the river basin in Bulgaria are carried out by various authors (Kirin et al., 2013; Kirin et al., 2014; Kuzmanova et al., 2019; Chunchukova & Kirin, 2020; Chunchukova et al., 2020; etc.). *Cobitis elongata*; *Cobitis taenia* and *Sabanejewia bulgarica* (syn. *Cobitis bulgarica* Drensky, 1928; *Cobitis aurata bulgarica* Drensky) are among the species poorly studied for parasites. Helminthological studies on *S. bulgarica* from the Bulgarian section of the Danube River are scarce (Margaritov, 1966; Kakacheva-Avramova, 1977; Kakacheva-Avramova et al., 1978). Studies on the parasite fauna of spined loach and *C. elongata* from the Bulgarian section of the river are lacking.

The present study aims to provide new data on the helminths of three fish species belonging to the Cobitidae family from the freshwater ecosystem of the Danube River in Bulgaria.

MATERIALS AND METHODS

For the period 2019-2021, 3 species of fish were collected - *Cobitis elongata* Heckel & Kner, 1858; spined loach, *Cobitis taenia* Linnaeus, 1758 and *Sabanejewia bulgarica* Drensky, 1928, caught from two places along the Danube River in the area of Kudelin and Koshava villages (marked as biotopes), Vidin Province, Northwestern Bulgaria. Kudelin biotope (44°12'07.9"N, 22°41'28.2"E) is located on the right bank of the Danube River, shortly after the river enters Bulgarian territory; about 35 km from the town of Vidin. Koshava biotope (44°03'59.9"N, 23°02'10.2"E) is also located on the right bank of the Danube River; about 20 km from the town of Vidin (Figures 1-2).

Fish were collected in accordance with the requirements of the Executive Agency for Fisheries and Aquaculture for catching fish for scientific research purposes. Fish species are represented by Vostradovsky (1973); Karapetkova & Zhivkov (2006); Kottelat & Freyhof (2007). In the field, immediately after capture, metric data - total length (TL) and maximum height (MH) of the body in centimeters; body weight (BW) in grams was determined on each of the fish specimens (Table 1).

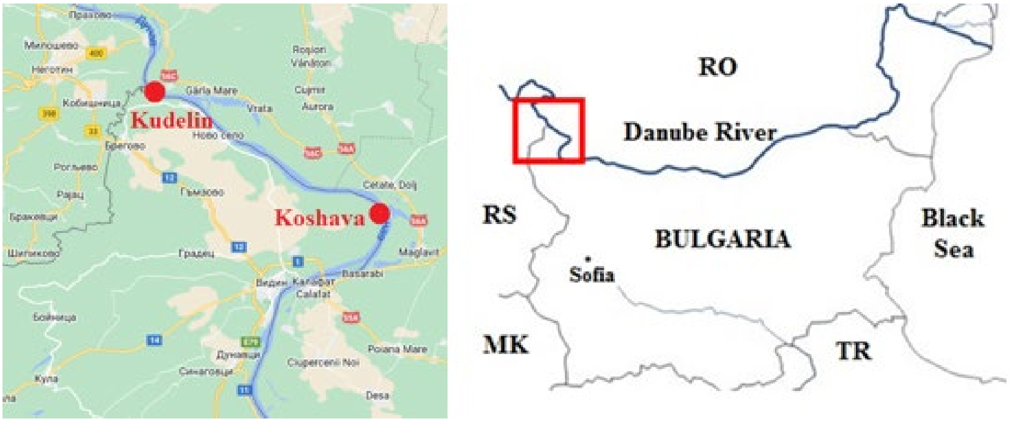

Figure 1. Location of Kudelin and Koshava biotopes along the Danube River, Vidin Province, Bulgaria (https://www.google.bg/maps/place/Видин)

Figure 2. Views from Danube River, Kudelin and Koshava biotopes; left to right (author's photos)

Table 1. Metric data (TL, MH, BW) of the examined specimens *Cobitis elongata*, *Cobitis taenia*

Danube River		TL (cm)	MH (cm)	BW (g)
***Cobitis elongata* N = 4**	**min-max**	5.3-8.9	0.6-1.3	1-2
	Mean±SD	7.58±1.57	0.95±0.29	1.50±0.58
***Cobitis taenia* N = 6**	**min-max**	6.5-9	1-1.4	1-4
	Mean±SD	8.02±0.90	1.23±0.20	2.83±1.33

A total of 11 specimens from the three fish species were examined by the method of the complete helminthological autopsy of the organs (Zashev & Margaritov, 1966; Moravec, 2013).

From the representatives of class Trematoda and class Nematoda, permanent and temporary microscopic preparations were prepared, respectively (Dubinina, 1948; Zashev & Margaritov, 1966; Moravec, 2013).

A microscope "XS-213" China was used to determine the type of helminths. The taxonomic affiliation of the isolated parasites was determined (by Bauer (Ed.), 1987; Moravec, 2013; and others).

RESULTS AND DISCUSSIONS

Fish species

Three freshwaters, demersal fish species, inhabiting the Danube River and its tributaries were studied. *C. elongata* prefers rivers with a moderate to fast current and a sandy bottom. The body is up to 17 cm long, and the weight - is up to 20 g. *C. taenia* inhabits slow-flowing and clean waters with a sandy bottom. The body length of the species reaches up to 15 cm, and the weight - is up to 15 g. The lifespan is 3-4 years. Spawns in the spring. It has a slow growth rate. *S. bulgarica* prefers rivers with fast currents and gravel bottoms. The species has a body length of up to 10 cm and a weight of up to 25 g. It spawns in the spring, entering the mouths of some of the Danube tributaries (Ogosta, Iskar, Vit, Osam, and Yantra rivers) to breed. The diet of the three studied fish species consists of benthic invertebrates. The species have no economic importance (Karapetkova & Zhivkov, 2006; Golemanski, 2011).

The three studied fish species are included in the IUCN Red List with the category "LC = Least Concern", as well as in the Biological Diversity Act in Bulgaria (Annex II). *C. elongata* and spined loach are included in the Bern Convention (Annex III). The spined loach is included in the Habitats Directive (Annex II). *S. bulgarica* is included in the Red Book of Bulgaria with the category "VU = Vulnerable" (Convention on the conservation of European wildlife and natural habitats, 1982; Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora, 1992; Biological Diversity Act, 2002; Freyhof & Brooks, 2011; Golemanski, 2011; IUCN, 2023).

Helminthological studies

The infection was found with two types of helminths - *Asymphylodora tincae* (Modeer, 1790) Lühe, 1909 (class Trematoda) and *Pseudocapillaria tomentosa* (Dujardin, 1845) Moravec, 1987 (class Nematoda) (Table 2). Infection was not found in six specimens of *C. taenia* from Koshava biotope and in two specimens of *C. elongata* from Kudelin biotope. Common helminth species were not found for the two infected loach species.

Table 2. Taxonomic position, synonyms, localization, season, hosts, minimum and maximum value of infection of *Asymphylodora tincae* and *Pseudocapillaria tomentosa*

Helminth species	***Asymphylodora tincae* (Modeer, 1790) Lühe, 1909**	***Pseudocapillaria tomentosa* (Dujardin, 1845) Moravec, 1987**
Taxonomic position	Family Monorchidae Odhner, 1911 Genus *Asymphylodora* Looss, 1899	Family Capillariidae Railliet, 1915 Genus *Pseudocapillaria* Freitas, 1959
Synonyms[1, 2]	*Asymphylodora perlata* (von Nordmann, 1832) Looss, 1899; *Distoma perlatum* von Nordmann, 1832; *Distoma tincae* (Modeer, 1790) Rudolphi, 1809; *Fasciola tincae* Modeer, 1790	*Capillaria amurensis* Finogenova, 1967; *C. tuberculata* (Linstow, 1914) Lewaschoff, 1929; *C. bakeri* Mueller & Van Cleave, 1932; *C. catostomi* Pearse, 1924; *C. gobionina* Lomakin, 1971; *C. leucisci* Hesse, 1923; *C. lewaschoffi* Heinze, 1993; *C. pseudorasborae* Wang, Zhao & Chen, 1978; *C. rutili* Zakhvatkin & Azheganova, 1940; *C. ugui* Yamaguti, 1941; *Skrjabinocapillaria elopichthydis* Wang, 1982; *Trichosoma brevispiculum* Linstow, 1873; *Tr. cyprini* Diesing, 1851; *Tr. tomentosum* Dujardin, 1843
Localization	intestine	intestine (especially in the distal part)
Season	spring	spring
Hosts	*Sabanejewia bulgarica*	*Cobitis elongata*
Minimum and maximum value of infection	2	1

[1]WoRMS (2022). *Asymphylodora tincae* (Modeer, 1790) Lühe, 1909. Accessed at: https://www.marinespecies.org/aphia.php?p=taxdetails&id=744986 on 2022-11-11

[2]Nemys eds. (2022). Nemys: World Database of Nematodes. *Pseudocapillaria (Pseudocapillaria) tomentosa* (Dujardin, 1843) Lomakin & Trofimenko, 1982. Accessed through: World Register of Marine Species at: https://www.marinespecies.org/aphia.php?p=taxdetails&id=991382 on 2022-11-11

As. tincae is determinated as a specific parasite of *Tinca tinca* (Linnaeus, 1758), but it has also been reported for the definitive hosts *Abr. brama*; *R. rutilus*, and others. The species is distinguished by a one-year cycle of development and has intermediate hosts - the snails *Bithynia tentaculata* (Linnaeus, 1758) and *Radix auricularia* (Linnaeus, 1758) (Bykhovskaya-Pavlovskaya et al., 1962; Gaevskaya et al., 1975; Kakacheva-Avramova, 1983; Bauer, 1987). Definitive hosts of *Ps. tomentosa* are freshwater fish of the families Cyprinidae, Balitoridae, Cobitidae, Siluridae, Blenniidae, Gobiidae, Percidae, Lotidae, Anguillidae, Esocidae, and others. Intermediate hosts are freshwater oligochaetes (*T. tubifex*, *Limnodrilus hoffmeisteri* Claparède, 1862, *Lumbricus variegatus* (Müller, 1774), and others) and others (Bauer, 1987; Moravec, 2013).

In one of the two examined specimens *C. elongata* from the Danube River (Koshava biotope) one specimen *Ps. tomentosa* was found with mean intensity MI = 1.00; mean abundance MA = 0.50 and prevalence P% = 50.00. From one specimen *S. bulgarica* from the Danube River (Kudelin biotope) two specimens *As. tincae* were isolated. Trematode *As. tincae* had equal mean intensity and mean abundance (MI = MA = 2.00), as well as prevalence P% = 100.00. *As. tincae* stood out by higher ecological indices.

The parasite fauna of the three investigated fish species is poorly studied. *C. elongata* has been reported as a host of *Allocreadium transversale* (Rudolphi, 1802) Odhner, 1901 from the Danube River basin in Bulgaria (Vit River) (Šmiga et al., 2020). *C. taenia* has been reported as a host of *Diplostomum spathaceum* (Rudolphi, 1819) Olsson, 1876, *Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908, and others from the Danube River basin in Serbia (Djikanovic et al., 2011). *S. bulgarica* has been reported as the host of *Nicolla skrjabini* (Iwanitzky, 1928) Dollfus, 1960 (syn. *Crowcrocaecum skrjabini* (Iwanitzky, 1928) Skrjabin & Koval, 1956) (Margaritov, 1966; Kakacheva-Avramova, 1977; Kakacheva-Avramova et al., 1978), *P. laevis* (Margaritov, 1966; Kakacheva-Avramova et al., 1978), Caryophyllaeidae G. sp., Acanthocephala G. sp. (Margaritov, 1966), and others from the Bulgarian section of the Danube River.

The trematode *As. tincae* identified in the present study was reported in *T. tinca* from the Lake Balaton part of the Danube River basin in Hungary (Molnár & Székely, 1995); in *T. tinca* and *R. rutilus* from rivers on the territory of Serbia (Djikanovic et al., 2011). The species was also discovered in *Abr. brama* from the Bulgarian section of the Danube River (Vetren biotope) (Chunchukova et al., 2016), as well as in *Scardinius erythrophthalmus* (Linnaeus, 1758) from the Srebarna Lake (Margaritov, 1959). The other helminth in this study - the nematode *Ps. tomentosa* was reported in *Barbus barbus* (Linnaeus, 1758) from the Hungarian section of the Danube River (Moravec et al., 1997); in *Ponticola kessleri* (Günther, 1861) (syn. *Neogobius kessleri* Günther, 1861) from the Slovak section of the river (Ondračková et al., 2009; Ondračková et al., 2010); in *Neogobius melanostomus* (Pallas, 1814) from the Austrian section of the Danube River (Ondračková et al., 2010); in *Carassius carassius* (Linnaeus, 1758) from the Danube River basin in Serbia (Djikanovic et al., 2011); in *Carassius gibelio* (Bloch, 1782) and *Pseudorasbora parva* (Temminck & Schlegel, 1846) from reservoirs in Moldova (Gologan, 2020); in *P. kessleri* (Ondračková et al., 2006; Ondračková et al., 2010) and *B. barbus* (Nachev, 2010) from the Bulgarian section of the Danube River.

CONCLUSIONS

As a result of the conducted ecologohel minthological research, *Asymphylodora tincae* (Modeer, 1790) Lühe, 1909 is reported for the first time as a helminth of *S. bulgarica* (Kudelin biotope), and *Pseudocapillaria tomentosa* (Dujardin, 1845) Moravec, 1987 is reported for the first time as a helminth of *C. elongata* (Koshava biotope). The received ecological indices were low, but *As. tincae* stood out with higher ecological indices. *C. elongata* is a new host for *Ps. tomentosa* in Bulgaria. *Ps. tomentosa* has not been reported by *C. elongata* from the Danube River and the river basin from other countries and Bulgaria. *S. bulgarica* is a new host for *As. tincae*. *As. tincae* has not been reported by *S. bulgarica* from the Danube River and its basin, both in other countries and in Bulgaria. Koshava and Kudelin biotopes are new habitats for the found helminth species of *C. elongata* and *S. bulgarica*, respectively.

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ECOPARASITOLOGICAL STUDY OF SIX SPECIES OF FISH FROM THE BULGARIAN SECTION OF THE DANUBE RIVER

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Abstract

For the period 2019-2021, an ecoparasitological study of six fish species - grass carp (Ctenopharyngodon idella Valenciennes, 1844); silver carp (Hypophthalmichthys molitrix Valenciennes, 1844); pumpkinseed (Lepomis gibbosus Linnaeus, 1758); Prussian carp (Carassius gibelio Bloch, 1782); gudgeon (Gobio gobio Linnaeus, 1758); European bitterling (Rhodeus amarus Bloch, 1782) was conducted. The fish were caught from 3 biotopes (Koshava, Kudelin, Novo selo) from the upper section of the Danube River in Bulgaria. A total of two parasite species (Pomphorhynchus laevis Zoega in Müller, 1776) Porta, 1908 and Contracaecum sp. (larvae) were found in two of the investigated fish species. Four fish species were not infected. The ecological indices (mean intensity; mean abundance; prevalence) of parasites were calculated. Kudelin and Koshava biotopes are new habitats for the found helminth species of the infected fish species.

***Key words**: freshwater fish, helminths, Koshava, Kudelin, Novo selo.*

INTRODUCTION

Studies on the parasite fauna of *Ct. idella* from the Danube River and the river basin in other countries and Bulgaria are few (Kakacheva-Avramova et al., 1978; Oros & Hanzelová, 2009; Hanzelová et al., 2011). Different authors (Urdeş & Hangan, 2013; Đikanović et al., 2018b; Gologan, 2020; Stroe et al., 2022) have researched parasites of *H. molitrix* from the Danube River and its basin. Studies on helminths of *L. gibbosus* from the Danube River are lacking, but there are data on the species from the river basin (Djikanovic et al., 2011; Djikanović et al., 2018a; Gologan, 2020; and others), and from five locations, part of the Ukraine Black Sea drainages (Kvach et al., 2023). Few authors have studied the parasite fauna of *C. gibelio* (syn. *Carassius auratus* (Bloch); *Carassius auratus gibelio* (Bloch, 1782)) from the Danube River in Bulgaria (Kakacheva-Avramova et al., 1978; Atanasov, 2012), in Romania (Stroe et al., 2022), as well as from the river basin in other countries (Gologan, 2020; Vuić et al., 2022) and in Bulgaria (Shukerova, 2005; 2010). The data on the parasite fauna of *G. gobio* (syn. *Gobio gobio gobio* (Linnaeus, 1758)) from the Danube River (Margaritov, 1966; Kakacheva-Avramova et al., 1978) and the river basin (Kakacheva-Avramova, 1969; Kakacheva-Avramova & Menkova, 1978) in Bulgaria date from the second half of the last century. Helminthological studies on *G. gobio* were also conducted from the river basin on the territory of other countries (Djikanovic et al., 2011; Ondračková et al., 2021; and others). Research on helminths of *Rh. amarus* is scarce (Dávidová et al., 2008).

The purpose of the present study is to provide new data on the helminths of six fish species from the freshwater ecosystem of the Danube River in Bulgaria; as well as new data on the ecological indices of found endohelminth species.

MATERIALS AND METHODS

For the period 2019-2021, a total of 21 specimens from 6 species of fish were subjected to parasitological research. The fish were caught from three sites along the Danube River in the vicinities of the villages Kudelin, Novo selo, and Koshava (noted as biotopes), Vidin Province, Northwestern Bulgaria. Kudelin, Novo selo, and Koshava biotopes are located at 844, 833, and 807 river km, respectively (Figures 1-2).

The fish were caught according to fishing permits for scientific research purposes, issued by the Executive Agency for Fisheries and Aquaculture (EAFA) to the Ministry of Agriculture, Bulgaria. The ecoparasitological examination of the caught fish specimens (Zashev & Margaritov, 1966) was preceded by the determination of the species affiliation (Karapetkova & Zhivkov, 2006; Fröse & Pauly, 2022) and by the recording of basic metric data. Basic ecological indices were calculated, such as mean intensity (MI); mean abundance (MA), and prevalence (P%) (Bush et al., 1997).

Figure 1. Location of Kudelin, Novo selo, and Koshava biotopes from the Danube River, Northwestern Bulgaria (https://www.google.bg/maps/place/Видин)

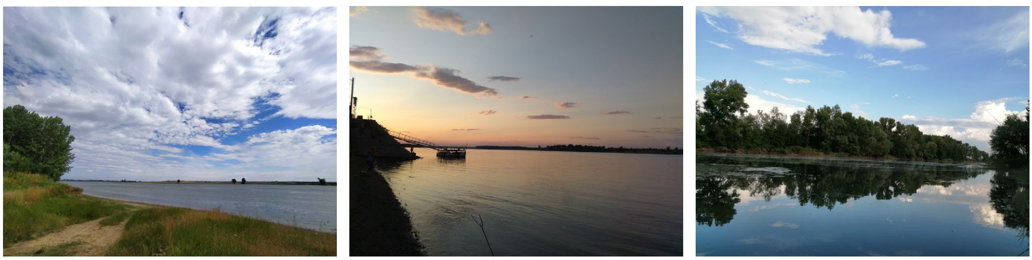

Figure 2. Views from Danube River, Kudelin, Novo selo, and Koshava biotopes; left to right (author's photos)

RESULTS AND DISCUSSIONS

The object of an ecoparasitological study were six fish species: grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844); silver carp, *Hypophthalmichthys molitrix* (Valenciennes, 1844); pumpkinseed, *Lepomis gibbosus* (Linnaeus, 1758); Prussian carp, *Carassius gibelio* (Bloch, 1782); gudgeon, *Gobio gobio* (Linnaeus, 1758); European bitterling, *Rhodeus amarus* (Bloch, 1782) from the freshwater ecosystem of the Danube River, differing in their way of feeding. All studied fish species are benthopelagic. *Ct. idella* has a body length of up to 130 cm and a weight of 35 kg. The species uses for food aquatic vegetation, detritus, insects, and invertebrates. The body length of *H. molitrix* reaches up to 1 m, and the weight is up to 16 kg. Food mainly includes phytoplankton and detritus. *L. gibbosus* is a small fish, with the weight of up to 250 g and a body length of up to 20 cm. It uses for food roe of other fish, zooplankton, and benthic invertebrates. *C. gibelio* has a body length of up to 45 cm and a weight of up to 3 kg. The diet of the species consists of plankton, benthic invertebrates, and plant food. *G. gobio* is a small fish with a body length of up to 20 cm and a weight of up to 100 g. It mainly uses aquatic vegetation and benthic invertebrates for food. *Rh. amarus* is a tiny fish, whose body reaches a length of up to 8 cm and weighs up to

15 g. The diet of the species includes plant food (algae), as well as crustaceans, insect larvae, and others (Karapetkova & Zhivkov, 2006; Fröse & Pauly, 2022). Four of the investigated fish species are included in the IUCN Red List, of which three species (*L. gibbosus*; *G. gobio* and *Rh. amarus*) are in the "LC" category, and one species (*H. molitrix*) is in the "NT" category. Only *Rh. amarus* is included in the Bern Convention, the Habitats Directive, and the Biological Diversity Act (Convention on the conservation of European wildlife and natural habitats, 1982; Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora, 1992; Biological Diversity Act, 2002; Freyhof & Brooks, 2011).

Ecoparasitological studies

For the period 2019-2021, a total of eight specimens of two herbivorous fish species were examined (1 specimen of *Ct. idella* from Kudelin biotope and 7 specimens of *H. molitrix* from Kudelin biotope); two specimens of one predatory fish species (*L. gibbosus* from Koshava biotope) and eleven specimens of three omnivorous fish species (9 specimens of *C. gibelio* form Kudelin and Novo selo biotopes, 1 specimen of *G. gobio* from Koshava biotope and 1 specimen of *Rh. amarus* from Kudelin biotope). In the study, an infection with a total of 2 species of helminths - *Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908 and *Contracaecum* sp. (larvae) was found (Table 1). Both helminth species were found in *C. gibelio*. One helminth species was found of *Ct. idella* - *P. laevis*. Four fish species (*H. molitrix*, *L. gibbosus*, *G. gobio* and *Rh. amarus*) were not infected. *P. laevis* is a common parasite for two of the studied fish species. Definitive hosts of *P. laevis* are freshwater fish species of the families Cyprinidae, Salmonidae, Percidae, Siluridae, etc. An intermediate host is *Gamarus pulex* (Linnaeus, 1758) (Petrochenko, 1956; Kakacheva-Avramova, 1983; Bauer (Ed.), 1987). Definitive hosts of *Contracaecum* sp. are waterfowl (*Ardea*, *Egretta*, *Podiceps*, *Phalacrocorax*), and intermediate hosts are copepods of the genera *Cyclops*, *Acanthocyclops*, *Macrocyclops*, *Mesocyclops*, *Eucyclops*, *Arctodiaptomus*, *Diaptomus* (Bauer (Ed.), 1987; Moravec, 2013).

Table 1. Taxonomic position, localization, season, hosts of *Pomphorhynchus laevis* and *Contracaecum* sp.

Helminth species	***Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908**	***Contracaecum* sp.**
Taxonomic position	**CLASS ACANTHOCEPHALA (RUDOLPHI, 1808)** Family Pomphorhynchidae Yamagiti, 1939 Genus *Pomphorhynchus* Monticelli, 1905	**CLASS NEMATODA RUDOLPHI, 1808** Family Anisakidae Skrjabin et Karokhin, 1945 Genus *Contracaecum* Railliet & Henry, 1912
Localization	intestine	in capsules on the serous membrane of the organs in the abdominal cavity of the fish
Season	Summer *C. gibelio*; autumn *Ct. idella*	summer
Hosts	*Carassius gibelio*, *Ctenopharyngodon idella*	*Carassius gibelio*

Ecoparasitological study of Ctenopharyngodon idella

During the examination of one specimen of *Ct. idella* from Kudelin biotope, one species of class Acanthocephala - *P. laevis*, was found. *P. laevis* had equal mean intensity and mean abundance (MI = MA = 2.00) (Table 2).

Table 2. Species diversity and ecological indices in the helminth community of *Ctenopharyngodon idella* from the Danube River

***Ctenopharyngodon idella* (N = 1/Kudelin) Parasite species**	**n**	**p**	**MI**	**MA**	**P%**	**R**
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	1	2	2.00	2.00	100.00	2

N - number of investigated fish; n - number of infected fish; p - number of fish parasites; MI - mean intensity; MA - mean abundance; P% - prevalence; R - range

Ecoparasitological study of Carassius gibelio

During the examination of 7 and 2 specimens of *C. gibelio* from Kudelin and Novo selo biotopes, respectively, infection was found in only one specimen from each biotope. One helminth species from class Acanthocephala - *P. laevis*, was found in the Prussian carp from Kudelin biotope, and one helminth species from class Nematoda - *Contracaecum* sp. was found in the Prussian carp from Novo selo biotope. Of the two detected helminth species, *Contracaecum* sp. had higher ecological indices (MI = 49.00, MA = 24.50 and P% = 50.00) (Table 3).

Table 3. Species diversity and ecological indices in the helminth community of *Carassius gibelio* from the Danube River

***Carassius gibelio* (N = 7/Kudelin) Parasite species**	**n**	**p**	**MI**	**MA**	**P%**	**R**
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	1	3	3.00	0.50	16.67	3
***Carassius gibelio* (N = 2/Novo selo) Parasite species**	**n**	**p**	**MI**	**MA**	**P%**	**R**
Contracaecum sp. (larvae)	1	49	49.00	24.50	50.00	49

N - number of investigated fish; n - number of infected fish; p - number of fish parasites; MI - mean intensity; MA - mean abundance; P% - prevalence; R - range

Oros & Hanzelová (2009); Hanzelová et al. (2011) examined for parasites *Ct. idella* from the Latorica River, part of the Danube River basin in Slovakia, and found the trematode *Sphaerostoma bramae* (Müller, 1776) Lühe, 1909 (syn. *Sphaerostomum bramae* Müller, 1776). *P. laevis* has been reported in grass carp from the Bulgarian section of the Danube River (Kakacheva-Avramova et al., 1978). Both parasites found in the present study in *C. gibelio* were reported either for the Danube River or the river basin in Bulgaria. *P. laevis* has been reported in *C. gibelio* from the Bulgarian section of the Danube River (Kakacheva-Avramova et al., 1978; Atanasov, 2012). While *Contracaecum* sp. is reported as *Contracaecum microcephalum* (Rudolphi, 1809) of Prussian carp from Srebarna Lake (Shukerova, 2005; 2010). Stroe et al. (2022) studied the parasite fauna of *C. gibelio* and *H. molitrix* from the Romanian section of the Danube River (Brăila station). The authors reported Trichodina sp., *Dactylogyrus vastator* (Nybelin, 1924) of Prussian carp, and *Diplostomum spathaceum* (Rudolphi, 1819) of silver carp. Vuić et al. (2022) reported *Contracaecum* larvae of Prussian carp from Lake Sakadaš, part of the Danube River basin in Croatia (Tables 4-5).

Table 4. Distribution of the found helminths (in the present study) of *Ctenopharyngodon idella* from the Danube River and its basin

Biotopes / Helminth species	Kudelin biotope	Danube River in other countries	Danube River Basin in other countries	Danube River in Bulgaria	Danube River Basin in Bulgaria
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	+	-	-	+	-

Table 5. Distribution of the found helminths (in the present study) of *Carassius gibelio* from the Danube River and its basin

Biotopes / Helminth species	Kudelin biotope	Novo selo biotope	Danube River in other countries	Danube River Basin in other countries	Danube River in Bulgaria	Danube River Basin in Bulgaria
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	+	-	-	-	+	-
Contracaecum sp.	-	+	-	+	-	+

According to the obtained results, the way of feeding is not determining by the infection. The mean intensity of the intermediate hosts in the studied biotopes has a predominant influence.

CONCLUSIONS

In the study of six fish species, it was found that only two species (*C. gibelio* and *Ct. idella*) were infected. The intermediate hosts of the found helminth species, rather than the diet of the hosts, are of predominant importance for the infection. When comparing the ecological indices of *P. laevis* from the two studied fish species, the highest prevalence was found for *P. laevis* of grass carp (Kudelin biotope) - P% = 100.00. In Prussian carp (Kudelin and Novo selo biotopes), higher mean intensity and mean abundance were established for *Contracaecum* sp. (MI = 49.00 and MA = 24.50). Kudelin and Novo selo biotopes are new habitats for the found helminth species of grass carp and Prussian carp.

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